Effects of Sarcolipin Ablation on Mitochondrial Enzyme Adaptations to Exercise Training

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Anton Trinh

ABSTRACT

Changes in intracellular Ca^{2+} ($[Ca^{2+}]_f$) and high-energy phosphates are known to induce adaptive changes in skeletal muscle during endurance exercising training, including mitochondrial biogenesis. Levels of $[Ca^{2+}]_f$ are regulated by sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCAs) which are further regulated by sarcolipin (SLN), through a reduction in the apparent affinity of SERCAs for Ca^{2+}. Furthermore, SLN reduces the efficiency of Ca^{2+} transport by SERCAs supporting a thermogenic role for SLN in skeletal muscle. Thus, it is possible SLN ablation could reduce Ca^{2+} and metabolic signaling during exercise training and attenuate increases in mitochondrial content. To investigate the potential role of SLN in the exercise-induced adaptive response of skeletal muscle, mice devoid of SLN (SLNKO) underwent endurance training for 8 weeks and were compared to WT controls. Maximal oxygen uptake ($\dot{V}O_2$ max) was measured with an exercise stress test while mitochondrial content was assessed through measurement of protein expression and maximal enzyme activities of several mitochondrial enzymes in soleus and extensor digitorum longus (EDL) muscles, which express high and low levels of SLN, respectively. All data were analyzed using a two-way analysis of variance (ANOVA) and student t-tests were conducted on enzyme data. $\dot{V}O_2$ max was found to not be significantly altered with exercise training in either genotype. Exercise training significantly increased the contents of adenine nucleotide translocase (ANT), cytochrome-*c* (cyt-*c*) and cytochrome-*c* oxidase subunit IV (COXIV) in soleus independent of genotype. Likewise, exercise training significantly increased cyt-*c* and COXIV expression ($P<0.04$), while increases in ANT expression were not significant ($P=0.13$) in the EDL. Two-way ANOVAs of mitochondrial enzymes

in soleus revealed an interaction existed for succinate dehydrogenase (SDH) where its activity was increased only in the SLNKO mice ($P<0.02$). In comparison, exercise training significantly elevated activities of cytochrome *c* oxidase (COX) and citrate synthase (CS) activities ($P<0.02$) but not β-hydroxyacyl-CoA dehydrogenase (β-HAD; $P=0.08$), independent of genotype. Upon closer examination using student t-tests, it was determined that exercise training induced greater increases in COX and CS activity in SLNKO compared to WT controls ($P<0.02$), similar to and consistent with SDH data. In EDL, only SDH activity increased following exercise training, an effect that was independent of genotype. In conclusion, these data suggest that SLN ablation does not attenuate exercise-induced mitochondrial adaptations and may increase mitochondrial enzyme adaptations to exercise training in slow-twitch muscle. Further examination of the effects of SLN on Ca^{2+} and metabolic signaling may provide mechanisms explaining the results of this thesis.

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DEDICATION

This thesis is dedicated to my family for their love, support and encouragement to strive higher and do great things. This journey has been long and at times difficult, but you have shown me how to succeed without compromise of character or integrity.

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Thesis Overview

It is well known that endurance exercise training induces an adaptive response within skeletal muscle to increase oxidative capacity (Holloszy and Booth, 1976). Only recently we have been able to identify several muscle contraction signals and corresponding signaling pathways involved in the regulation of mitochondrial gene expression with exercise training. In the exercising skeletal muscle, increases in the [AMP]/[ATP] ratio and elevations in intracellular Ca^{2+} ($[Ca^{2+}]_f$) can stimulate mitochondrial biogenesis through induction of the PGC-1α expression and activity, resulting in mitochondrial gene expression (Joseph *et al*., 2006). PGC-1α is considered to be a major regulator of mitochondrial biogenesis in skeletal muscle (Lin *et al*., 2005; Lira *et al*., 2010).

During exercise, decreases in ATP coinciding with increases in AMP, activate AMP-protein kinase (AMPK), an enzyme regulating energy status in skeletal muscle (Hardie and Sakamoto, 2006). Activation of AMPK induces metabolic changes including mitochondrial biogenesis, gene expression of proteins regulating glucose and fatty acid (FA) uptake, and stimulation of FA oxidation (Jørgensen *et al*., 2006). Studies utilizing pharmacological agents such as AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide) or transgenic animals, have demonstrated the importance of AMPK signaling in mitochondrial biogenesis (Jørgensen *et al*., 2006). Elevation in $[Ca^{2+}]_f$ resulting from increased motor activation during exercise promotes the activation of two primary Ca^{2+}-dependent transcriptional pathways promoting mitochondrial biogenesis. Increases in Ca^{2+} transient duration and amplitude represent primary exercise signals that are decoded by Ca^{2+}-dependent signaling molecules such as Ca^{2+}/calmodulin-dependent

kinases (CaMKII and CaMKIV) and calcineurin (CnA). Studies using cultured myocytes and transgenic animals have shown that increases in $[Ca^{2+}]_f$ induce expression of mitochondrial enzymes concomitant with transcription factors promoting mitochondrial biogenesis (Chin, 2005).

The essential role of Ca^{2+} as an intracellular signaling molecule in numerous cellular processes is well established, particularly in excitation-contraction (EC) coupling (Rasmussen, 1986; Berchtold *et al*., 2000). The rate of contraction and relaxation of a muscle is governed by the release and uptake of Ca^{2+} by the sarcoplasmic reticulum (SR). Uptake of Ca^{2+} by sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCAs) promotes relaxation and the activity of SERCAs can be altered by the SERCA regulatory protein, sarcolipin (SLN; Tupling, 2009). Specifically, SLN reduces the apparent affinity of SERCA for Ca^{2+} and reduces the optimal coupling ratio from 2:1 (Ca^{2+}:ATP hydrolyzed). Experiments utilizing cardiomyocytes and skeletal muscle have demonstrated the significant role of SLN in regulating SERCA. First, the absence of SLN increases SERCA efficiency and reduces skeletal muscle metabolism when compared to wild type (WT) counterparts (Bombardier *et al*., unpublished; Bal *et al*., 2012). Secondly, ablation of SLN alters Ca^{2+} transients and enhances contractility in both cardiomyocytes (Gramolini *et al*., 2006; Bhupathy *et al*., 2007; Bhupathy *et al*., 2009), and skeletal muscle (Tupling *et al*., 2011). The ability of SLN to regulate skeletal muscle metabolism and Ca^{2+} transients suggests that SLN may modulate the adaptive response of skeletal muscle to increase oxidative capacity with endurance exercise training. The role of SLN in exercise training-induced mitochondrial biogenesis is unknown. Thus it is of interest to determine if SLN ablation has an effect on exercise-induced mitochondrial biogenesis in

skeletal muscle. Since SLN ablation would be expected to reduce metabolic signals that activate AMPK and increase the amplitude and decrease the duration of Ca^{2+} transients, it is hypothesized that exercise- induced mitochondrial biogenesis would be attenuated in SLN knock-out mice (SLNKO) compared to wild type (WT) controls.

Introduction

Exercise Training and Mitochondrial Biogenesis

It has been long known that endurance exercise training is a potent inducer of mitochondrial biogenesis and increases oxidative capacity in skeletal muscle (Holloszy and Booth, 1976; Coffey and Hawley, 2007; Hood, 2009; Gunderson, 2011). Furthermore, differences in oxidative capacity between fibre types could be attributed to differences in oxidative genes expressed (Chin, 2005) which is influenced by the pattern of muscle excitation (Pette and Staron, 1997). Holloszy (1967) first demonstrated that endurance exercise training resulted in greater fatigue resistance that occurred concomitant with elevated mitochondrial content and enzyme activities. Since then, extensive research has gone into identifying the mechanisms by which exercise increases skeletal muscle oxidative capacity.

Increased mitochondrial content accompanying chronic contractile activity is commonly referred to as mitochondrial biogenesis. This process is complex and requires coordinated expression of nuclear and mitochondrial encoded genes, which is regulated by several important transcriptional factors (Joseph *et al*., 2006). One such protein mediating these adaptations is the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), a transcriptional co-activator that has been identified as the "master" regulator of mitochondrial biogenesis (Lin *et al*., 2005; Lira *et al*., 2010). Downstream targets of PGC-1α include the nuclear respiratory factors 1 and 2 (NRF-1, NRF-2) and mitochondrial transcription factor A (TFAM), which regulate the expression of several nuclear encoded electron transport chain (ETC) genes and mitochondrial DNA (mtDNA), respectively (Scarpulla, 2002; Baar, 2004).

Multiple signaling pathways have been implicated in regulation of PGC-1α expression and/or activity at transcriptional and post-translational levels. These signaling pathways are activated through the concerted action of intramuscular metabolic signals generated during contractile activity. Decreased levels of high-energy phosphates (Hardie and Sakamoto, 2006), generation of reactive oxygen species (ROS; Powers *et al*., 2011), and increased levels of $[Ca^{2+}]_f$ (Chin, 2005), which has been suggested to be fundamental for inducing skeletal muscle mitochondrial biogenesis, have been implicated in the adaptive response of skeletal muscle to endurance exercise training. The remainder of this thesis will focus on the role of Ca^{2+} and metabolic signaling in mitochondrial biogenesis.

The Role of Ca^{2+} in Skeletal Muscle Mitochondrial Biogenesis

The intensity of muscle loading during exercise is associated with distinct patterns of muscle excitation (Pette and Staron, 1997) and oscillations of $[Ca^{2+}]_f$. In addition to eliciting contraction, the duration and amplitude of Ca^{2+} transients determine genes expressed. This link between muscle excitation and gene transcription is called excitation transcription coupling (Chin, 2004). Numerous studies have demonstrated increased $[Ca^{2+}]_f$ is fundamental in activating transcriptional responses to increase mitochondrial content in cultured myocytes and transgenic animals where Ca^{2+} handling is altered (Chin, 2005). Changes in the amplitude and temporal distribution of Ca^{2+} are decoded by at least two major Ca^{2+}-signaling molecules; calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase, and Ca^{2+}/calmodulin-dependent protein kinases (CaMKs; Chin, 2004) (**Figure 1**). Calcineurin is activated by sustained, low amplitude elevations in

$[Ca^{2+}]_f$, typical of slow-twitch (type I) fibres activated by low frequencies (10-30 Hz; Chin, 2004). In contrast, CaMKs are sensitive to greater amplitudes and frequencies of Ca^{2+} transient oscillations, which is representative of fast-twitch (type II) fibre activation. A number of CaMK isoforms exist; however, CaMKII appears to be predominantly expressed in skeletal muscle (Chin, 2005). CaMKII activity and expression is rapidly increased at the onset of exercise and sustained throughout the duration of activity (Rose *et al*., 2006). Furthermore, the magnitude of CaMKII activation is dependent on the intensity of exercise (Rose *et al*., 2006) and remains elevated following exercise training (Rose *et al*., 2007). Thus, repeated elevations of $[Ca^{2+}]_f$ occurring with exercise, increase the activation of these Ca^{2+} signaling molecules.

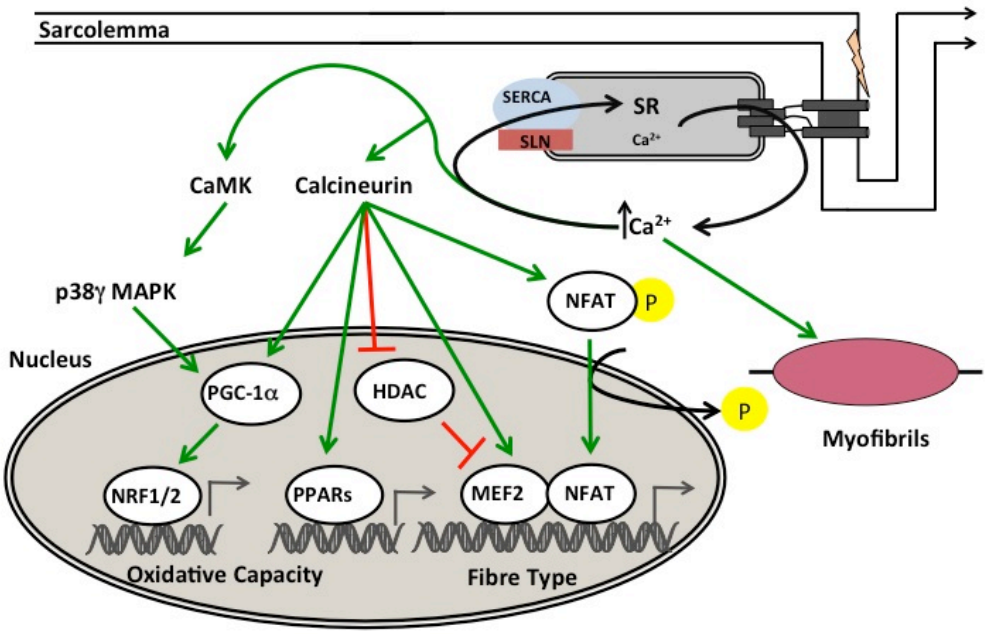

Figure 1. Ca^{2+}-dependent transcriptional signaling (adapted from Chin, 2005). Abbreviations: Ca^{2+}/calmodulin-dependent protein kinases (CaMK), p38γ mitogen activated protein kinase (p38γ MAPK), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), histone deacetylase (HDAC), nuclear factor of activated T-cells (NFAT), nuclear respiratory factors (NRF) 1 and 2, peroxisome proliferator-activated receptors (PPARs) and myocyte enhancer factor-2 (MEF2).

Initial experiments showed continuous treatment of L6 myotubes with a Ca^{2+} ionophore increased mRNA expression of cytochrome-*c* (cyt-*c*; Freyssenet *et al*., 1999) and protein expression of mitochondrial enzymes, δ-aminolevulinate synthase (ALAS) and cytochrome oxidase subunit 1 (COX1; Ojuka *et al*., 2002). However, continuous exposure to a Ca^{2+} ionophore had a detrimental effect on cell viability and caused a loss of myotubes (Ojuka *et al*., 2002). To circumvent this, intermittent treatment of L6 myotubes (5h/day) with other Ca^{2+} mobilizing agents (ionomycin, caffeine or W7) increased protein expression of ALAS, COX, and cyt-*c* without myocyte loss (Ojuka *et al*., 2002). Furthermore, intermittent caffeine exposure of L6 myotubes increased protein expression of PGC-1, NRF-1, NRF-2, and TFAM, an adaptation that was prevented by dantrolene (RyR blocker) or EGTA (Ca^{2+} chelating agent; Ojuka *et al*., 2003). The exposure of cultured myocytes to Ca^{2+} agonists, is intended to mimic exercise conditions where $[Ca^{2+}]_f$ would be elevated in skeletal muscle. Increases in mitochondrial protein expression occurring with rising $[Ca^{2+}]_f$ in cultured myocytes demonstrate the importance of Ca^{2+} in mediating mitochondrial biogenesis.

One limitation of cell culture studies is that the levels of $[Ca^{2+}]_f$ do not reflect physiological conditions. I*n vivo* evidence of Ca^{2+} mediated mitochondrial biogenesis has come from several studies involving transgenic animal models. For example, mice lacking parvalbumin (PV), a Ca^{2+} buffering protein expressed exclusively in fast twitch muscle, display a slower rate of $[Ca^{2+}]_f$ decay compared to WT mice (Chen *et al*., 2001). Consequently, fast-twitch muscle displays increased fatigue resistance, mitochondrial fractional volume, and expression of mitochondrial proteins (Chen *et al*., 2001; Racay *et* al., 2006). The lack of PV allows $[Ca^{2+}]_f$ to remain elevated and activate calcineurin

and/or CaMKs resulting in transcriptional signaling to increase mitochondrial content. Conversely, overexpression of PV in mouse slow-twitch muscle decreased twitch and relaxation times which was associated with reduced calcineurin activity and lowered oxidative capacity (Chin *et al*., 2003). Both PV transgenic models confirmed that the oxidative capacity of skeletal muscle is modulated by physiological alterations to Ca^{2+} regulatory proteins and their consequent alterations to $[Ca^{2+}]_f$.

Further support for Ca^{2+}-dependent mitochondrial biogenesis has come from transgenic mouse models where Ca^{2+} signaling molecules have been altered. Overexpression of a constitutively active form of CaMKIV increased both mitochondrial content and the proportion of type I fibres, and increased resistance to fatigue in skeletal muscle (Wu *et al*., 2002). However, CaMKIV is not expressed in skeletal muscle, whereas CaMKII appears to be the dominant isoform (Wu *et al*., 2002; Rose *et al*., 2006). Consistent with the lack of CaMKIV in skeletal muscle, mice without CaMKIV responded to long-term voluntary running with similar increases compared to WT mice in oxidative and slow-fibre specific proteins (Akimoto *et al*., 2004). In another example, mice expressing activated calcineurin displayed enhanced lipid oxidation (Long *et al*., 2007) and greater skeletal muscle oxidative capacity (Jiang *et al*., 2010). The resultant increase in skeletal muscle mitochondrial content and oxidative capacity occurring with manipulation of calcineurin and CaMK expression in transgenic mice provide further evidence that activation of Ca^{2+} signaling is important for mitochondrial biogenesis.

The Role of AMPK in Skeletal Muscle Mitochondrial Biogenesis

AMPK is an energy-sensing enzyme that is activated during exercise (Hardie and Sakamoto, 2006; Jørgensen *et al*., 2006; Freyssenet, 2007). More specifically, AMPK is activated when the concentration of high-energy phosphates are decreased or through phosphorylation by upstream kinases such as liver kinase B1 (LKB1) and CaMK kinase β (CaMKK-β; Hardie and Sakamoto, 2006). In addition to responding to metabolic stress, AMPK can also be activated by increased ROS production (Hood, 2009; Lira et al., 2010). Activation of AMPK in skeletal muscle induces metabolic changes to glucose and fatty acid metabolism, and increases in mitochondrial gene expression (**Figure 2**; Hardie and Sakamoto, 2006; Jørgensen *et al*., 2006). Moreover, it has been demonstrated that activation of AMPK is highly responsive to exercise in a time and intensity dependent manner (Stephens *et al*., 2002; Chen *et al*., 2003). Thus, increases in the [AMP]/[ATP] ratio occurring in endurance exercise would activate AMPK signaling to reduce metabolic stress in skeletal muscle.

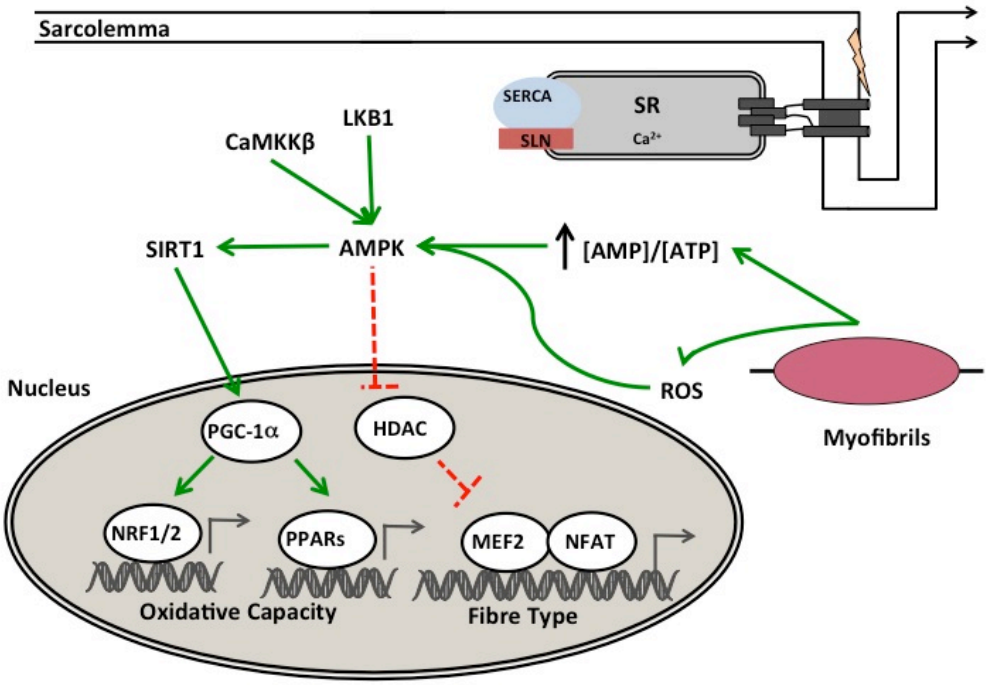

Figure 2. AMPK-dependent transcriptional signaling. Abbreviations: Ca^{2+}/calmodulin-dependent protein kinase β (CaMKK-β), liver kinase B1 (LKB1), sirtuin 1 (SIRT1), AMP-activated protein kinase (AMPK), peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), histone deacetylase (HDAC), nuclear factor of activated T-cells (NFAT), nuclear respiratory factors (NRF) 1 and 2, peroxisome proliferator-activated receptors (PPARs) and myocyte enhancer factor-2 (MEF2).

The role of AMPK signaling for mitochondrial biogenesis has come from numerous cell and animal studies utilizing pharmacological agents. Treatment of L6 myotubes with the AMPK agonist AICAR (5-aminoimadazole-4-carboxamide riboside) has been shown to increase expression of cyt-*c*, CS (Ojuka et al., 2002*a*) and PGC-1α (Irrcher *et al*., 2003). Likewise, administration of AICAR to mice induced increases in cyt-*c*, COX1 and CS expression *in vivo* (Jørgensen *et al*., 2007). Another drug, β-guanadinopropionic acid (β-GPA), has been used to mimic chronic energy deprivation by inhibiting creatine kinase in non-exercising muscle. Treatment with β-GPA in mice expressing a dominant-negative mutant of AMPK (DN-AMPK) failed to increase

mtDNA or protein expression of cyt-*c* and ALAS, whereas WT controls displayed significant increases in the aforementioned mitochondrial markers (Zong *et al*., 2002). These results provide evidence that activation of AMPK or imitation of high-energy phosphate depletion can increase mitochondrial content. Moreover, increases in PGC-1α expression (Irrcher *et al*., 2003) and promoter activity (Irrcher *et al*., 2008) have been demonstrated *in vivo* and *in vitro* during contractile activity. Recently, the activity of AMPK has been linked to the actions of sirtuin 1 (SIRT1), an enzyme responsible for deactylation of proteins including PGC-1α (Cantó *et al*., 2010; Yan *et al*., 2011), further demonstrating the importance of AMPK in regulating mitochondrial biogenesis. Specifically, AMPK was shown to enhance the activity of SIRT1 through increasing cellular levels of NAD^+ (Cantó *et al*., 2009). Thus, these studies demonstrate that increased AMPK signaling induces the expression and activity of transcriptional proteins involved in mitochondrial biogenesis.

Excitation - Contraction Coupling and Regulation of Ca^{2+}

Skeletal muscle EC coupling describes the processes linking an action potential to contraction of striated muscle. More specifically, it is the link between sarcolemmal depolarization and release of intracellular free Ca^{2+} ($[Ca^{2+}]_f$); Dulhunty, 2006). This process begins with the generation of an action potential by the α-motor neuron, which travels along the sarcolemmal membrane towards the transverse tubule (T-tubule). Located here are voltage sensitive dihydropyridine receptors (DHPRs), which physically interact with ryanodine receptors (RyRs) located adjacently on the sarcoplasmic reticulum (SR) to form Ca^{2+} release units. Changes in voltage induced by depolarization

are sensed by DHPRs triggering a conformational change. This results in opening of the RyRs of the SR and subsequent Ca^{2+} efflux into the cytosol (Rossi and Dirksen, 2005). Increased levels of $[Ca^{2+}]_f$ allows Ca^{2+} to bind troponin-C along the actin thin filament, which removes steric inhibition of tropomyosin, and exposes myosin-binding sites along actin. As a result, strong binding state crossbridge formation occurs between actin and myosin. The release of inorganic phosphate (P_i) and adenosine diphosphate (ADP) causes myosin crossbridge movement, producing contraction and generation of force. Relaxation occurs with termination of the action potential, closure of RyRs, and uptake of Ca^{2+} into the SR by the sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCAs; Meissner and Lu, 1995; Melzer *et al*., 1995; Stephenson *et al*., 1998).

The primary regulators of $[Ca^{2+}]_f$ in resting muscle are SERCA pumps (Wu and Lytton, 1993). These are SR integral membrane proteins comprised of a large cytoplasmic headpiece with three domains (actuator, nucleotide binding, and phosphorylation), ten transmembrane helices (M1-M10), and short luminal loops (Toyoshima and Inesi, 2004). Several SERCA isoforms exist within mammalian muscle; fast-twitch skeletal muscle predominantly expresses SERCA1a while slow-twitch skeletal muscle and cardiac muscle express SERCA2a (Wu and Lytton, 1993). The uptake of Ca^{2+} into the SR is an energy-dependent process due to a large Ca^{2+} gradient ($>10^4$) across the SR membrane (Rossi and Dirksen, 2005). Under optimal conditions, SERCAs can transport 2 Ca^{2+} ions into the SR for each ATP hydrolyzed. This coupling ratio (2:1) is in agreement with the two Ca^{2+} binding sites and one ATP binding site found on each SERCA molecule, as determined by X-ray crystallography (Rossi and Dirksen, 2005;

Toyoshima and Inesi, 2004). However, the coupling ratio is reduced under physiological conditions (de Meis, 2001b; Smith *et al*., 2001; Bombardier, 2010).

A SERCA reaction cycle begins with binding of 2 Ca^{2+} ions to a high affinity Ca^{2+} binding pocket found in the transmembrane domain facing the cytoplasm (Asahi *et al*., 2003; Morita *et al*., 2007). ATP binding to the nucleotide binding domain results in phosphorylation of SERCA. Subsequently, this induces conformational change of the transmembrane domain transforming the Ca^{2+} binding pocket to a state of low Ca^{2+} binding affinity as it faces the SR lumen (Lee, 2002). One cause of reduced SERCA coupling ratio is a premature release of Ca^{2+} to the cytoplasm during conformational changes, commonly referred to as slippage (Mall *et al*., 2006). This is a result of decreased Ca^{2+} affinity of SERCAs due to high luminal [Ca^{2+}] within the SR, or the presence of SERCA regulatory proteins such as sarcolipin (SLN; Odermatt *et al*., MacLennan *et al*., 2003; Tupling, 2009). SLN can physically interact with SERCAs and block the Ca^{2+} binding pocket within the transmembrane domain, thus altering Ca^{2+} binding affinity and causing slippage (Bhupathy *et al*., 2007). Ultimately, less Ca^{2+} is taken up into the SR and remains in the cytosol thereby modulating Ca^{2+} transients and potentially, activation of transcriptional signaling. Furthermore, slippage reduces the transport of Ca^{2+} and energy derived from ATP hydrolysis is converted to heat, thereby uncoupling the two processes (Smith *et al*., 2002). As a result, in the presence of SLN, a greater amount of ATP is required to transport Ca^{2+} into the SR and there is an increase in heat production. The significant role SLN has in Ca^{2+} uptake and skeletal muscle thermogenesis suggests it may be important in regulating Ca^{2+} and AMPK-mediated mitochondrial biogenesis, a hypothesis that will be tested in this thesis.

Regulation of SERCA by SLN

SLN is a small 31 amino acid SR integral membrane protein that physically interacts with SERCA and effectively inhibits its activity by reducing the apparent Ca2+ affinity of SERCA (Odermatt et al., 1998). Sequencing of SLN revealed it is comprised of a 7 amino acid N-terminal domain, a 19 amino acid transmembrane domain, and a 5 amino acid C-terminal domain protruding into the SR lumen (Odermatt *et al.*, 1997). SLN was originally identified as a low molecular weight proteolipid associated with SERCA1a of rabbit fast-twitch skeletal muscle (MacLennan *et al*., 1974). Since then, protein expression of SLN has been found to be both tissue and species specific (Vangheluwe *et al*., 2005; Babu *et al*., 2007). In small mammals such as mouse, SLN is highly expressed in tongue, diaphragm, soleus, red gastrocnemius (RG), and atria, and to a lesser extent in extensor digitorum longus (EDL), white gastrocnemius (WG), and ventricle of the heart. In larger mammals such as dog and rabbit, SLN expression was found in all skeletal muscles examined and atrium, but not in ventricle (Vangheluwe *et al*., 2005; Babu *et al*., 2007; Tupling *et al*., 2011).

Over the past three decades, SLN has emerged as an important regulator of SERCA activity. The physiological role of SLN has been well researched in cardiac muscle but less is known about its function in skeletal muscle. SLN regulates SERCA in part through its physical interactions with SERCAs Ca2+ binding sites (Asahi *et al*., 2003), reducing the apparent affinity for Ca^{2+} (Tupling *et al*., 2002; Tupling *et al*. 2011). The inhibitory effect of SLN was found to be reversible in cardiac muscle whereby SLN inhibition is relieved upon phosphorylation (Gramolini *et al*., 2006; Bhupathy *et al*., 2009). Regulation of SLN function in skeletal muscle has yet to be elucidated however

recent evidence has suggested that a similar mechanism exists (Tupling *et al*., 2011). Further research is needed to confirm whether phosphorylation/dephosphorylation processes in skeletal muscle regulate SLN.

SLN Expression Alters Ca^{2+} Transients and Muscle Contractility

The role of SLN in regulating SERCA has been well established. Despite this, the physiological role of SLN has only been investigated recently and is slowly being clarified. As a consequence of reduced Ca^{2+} affinity of SERCA, SLN was found to alter Ca^{2+} handling dynamics and muscle contractility (Tupling, 2009). Overexpression of SLN in cardiomyocytes reduced Ca^{2+} uptake and Ca^{2+} transient amplitude, resulting in impaired cardiac contractility (Babu *et al*., 2006; Gramolini *et al*., 2006). The reduction in Ca^{2+} uptake is thought to prolong Ca^{2+} transient duration and decrease SR refilling of Ca^{2+}, thereby diminishing subsequent Ca^{2+} release and contractile force. Likewise, SLN overexpression in slow-twitch skeletal muscle decreased peak twitch and tetanic force, reduced muscle contractility, and increased muscle fatigability (Tupling *et al*., 2002). Furthermore, in nebulin-free skeletal muscle, SLN expression was reported to be upregulated and was associated with lower maximal Ca^{2+} transport and longer half-relaxation time (Ottenheijm *et* al., 2008). In cardiac muscle, with an enhancement in Ca^{2+} uptake, SR refilling is increased which effectively results in greater Ca^{2+} release upon subsequent stimulation and greater contractility. Not surprisingly, ablation of SLN in atria increased the affinity of SERCA for Ca^{2+} resulting in increased Ca^{2+} transient amplitudes and enhanced contractility (Babu *et al*., 2007; Xie *et al*., 2012). Moreover, skeletal muscle devoid of SLN displayed increased Ca^{2+} uptake and enhanced relaxation

(Tupling *et al*., 2011). It is evident that SLN is a key regulator of $[Ca^{2+}]_f$, whereby it affects SR Ca^{2+} uptake and subsequently skeletal muscle contractility. Furthermore, SLN overexpression has been shown to reduce the amplitude and prolong the duration of Ca^{2+} transients thereby potentially impacting transcriptional signaling activated by exercise training. In particular, given the known role of SLN on Ca^{2+} handling, it is possible that SLN could exert an effect on Ca^{2+}-dependent signaling in mitochondrial biogenesis.

SLN Ablation Improves SERCA Energetic Efficiency

SLN is known to effectively inhibit SERCAs through reduction of the apparent affinity for Ca^{2+} (Odermatt *et al*., 1998) and as a result, uncouples ATP hydrolysis from the movement of Ca^{2+} (Smith *et al*., 2002). The uncoupling of ATP hydrolysis by SLN has been shown to increase the production of heat (Smith *et al*., 2002; Mall *et al*. 2006). Thus, in the absence of SLN, uncoupling is reduced and less energy is required to transport Ca^{2+} into the SR. In support of this, SLNKO mice have a higher SERCA coupling ratio in isolated soleus compared to their WT counterparts. Furthermore, SLNKO mice display lower whole body $\dot{V}O_2$ under sleeping and resting conditions (Bombardier *et al*., unpublished). Recently it has been demonstrated that SLNKO mice exhibit impaired muscle-based thermogenesis in skeletal muscle and are more susceptible to diet-induced obesity (Bal *et al*., 2012). The effect of SLN on skeletal muscle thermogenesis provides evidence that SLNKO mice are more energy efficient. Therefore, it might be expected that SLN influences the energy status of skeletal muscle during exercise and potentially could play a role in AMPK-mediated mitochondrial biogenesis.

Study Rationale

SLN is an important regulator of SERCAs, which is apparent by its effects on Ca^{2+} handling and contractility in cardiac and skeletal muscle. Furthermore, the effect of SLN on SERCA energetics suggests it is an important regulator of muscle thermogenesis and whole body metabolism. It is well recognized that elevated $[Ca^{2+}]_f$ and decreases in high energy phosphates are important signals activating mitochondrial biogenesis in skeletal muscle. Given the importance of $[Ca^{2+}]_f$ and metabolic signaling in exercise induced mitochondrial biogenesis, it is of interest to determine if SLN ablation would alter the adaptive response to exercise training. Previously it was shown that skeletal muscle oxidative metabolism did not differ between WT and SLNKO mice under sedentary or voluntary exercising conditions (Gamu, 2012); however, the exercise stimulus was too mild to elicit an adaptive response and reveal potential phenotype differences. This study expands on the earlier study by using a forced exercise training protocol to investigate if any phenotype differences in exercise induced mitochondrial biogenesis between trained WT and SLNKO mice exist. To investigate if SLN ablation alters the adaptive response to exercise training, an examination of oxidative capacity in WT and SLNKO mice in response to endurance exercise training was conducted.

Study Objectives and Hypotheses

Objectives

1) Determine if the adaptive increase in skeletal muscle mitochondrial content in response to endurance exercise training is altered in the soleus muscle of SLNKO mice in comparison to WT mice.
2) Determine if the adaptive increase in skeletal muscle mitochondrial content in response to endurance exercise training is altered in the EDL muscle of SLNKO mice in comparison to WT mice.

Hypotheses

1) Given that SLN ablation has been shown to reduce the duration and increase the amplitude of Ca^{2+} transients, it would be expected that a reduction in the duration of $[Ca^{2+}]_f$ oscillations would reduce the activation of transcriptional signaling. Therefore it is hypothesized that SLNKO mice would display an attenuated response to increasing mitochondrial content in soleus following endurance exercise training compared to WT mice.
2) Fast-twitch muscles, such as extensor digitorum longus, have low endogenous levels of SLN thus, it would be expected that no genotype differences in mitochondrial enzymes would exist between WT and SLNKO mice following endurance exercise training.

Methods

Experimental Animals

SLNKO mice (Babu *et al*., 2007) were generously donated by Dr. Muthu Periasamy from the Ohio State University and were used to establish a breeding colony at the University of Waterloo. SLNKO mice were crossbred with mice of a C57BL/6 background to create heterozygous SLN null (HET, +/-) breeding pairs. Breeding of HET mice yielded homozygous SLN knockout (SLNKO, -/-), heterozygous (HET, +/-), and homozygous wild type (WT, +/+) mice. Mouse genotype was determined at four weeks of age, where ear clippings were taken, digested, and DNA extracted using a PureLink DNA kit (Invitrogen). Approximately 50 ng of extracted DNA was added to a Taq DNA polymerase mixture (Fermentas) containing 3 mM $MgCl_2$, 200 µM dNTP, 10x Taq buffer (containing 750 mM Tris-HCL, 200 mM $(NH_4)_2SO_4$, and 0.1% (v/v) Tween 20), 1.5 µL Taq DNA polymerase, and 0.4 µM of appropriate forward and reverse primers (WT forward: 5'-TGT CCT CAT CAC CGT TCT CCT-3', WT reverse: 5'-GCT GGA GCA TCT TGG CTA ATC-3', SLNKO forward: 5'- GTG GCC AGA GCT TTC CAA TA-3', SLNKO reverse: 5'- CAA AAC CAA ATT AAG GGC CA-3'). Extracted DNA was placed in a thermal cycler (S1000 Thermal Cycler; Bio-Rad) and denatured at 94ºC for 3 min, followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 54ºC for 30 sec, and extension at 72ºC for 60 sec, finishing with a final extension at 72ºC for 7 min. Amplified target DNA was resolved using electrophoresis on a 1% agarose gel containing 0.013% ethidium bromide (BioShop), and identified under UV light using a bio-imaging system and GeneSnap software (Syngene).

At 3-4 months of age, WT and SLNKO male mice were separated into individual cages and housed in a temperature-controlled room under a reverse light/dark cycle (12/12 hr). Mice were given *ad libitum* access to water and standard rodent chow (22/5 Rodent Diet 8640; Harland-Tekland, Madison, WI). All animal experiments were approved by the University of Waterloo Animal Care Committee and performed in accordance with guidelines by the Canadian Council on Animal Care.

Treadmill Acclimation

Prior to initiation of the exercise-training program, mice were acclimated to a motor driven treadmill for one week (Exer -3/6 Treadmill; Columbus Instruments). The treadmill was divided into six lanes and a pulsed stimulus platform (200ms bursts with user adjustable rate) was used to provide a mild shock to promote running. Mice were acclimated over five consecutive days for 20 min each day. The first three days consisted of sitting on the treadmill for 5 min, followed by 15 min of running at 9 m/min at 0° incline. During the last two days of treadmill acclimation, mice sat on the treadmill for 5 min followed by running for 15 min at 9 m/min and a 5° incline.

Exercise Training

At the end of the acclimation period, WT and SLNKO male mice (n=36) were randomly assigned to either an exercise-training group or a control group (**Figure 3**, n=9 per group). Body weights of mice were monitored weekly.

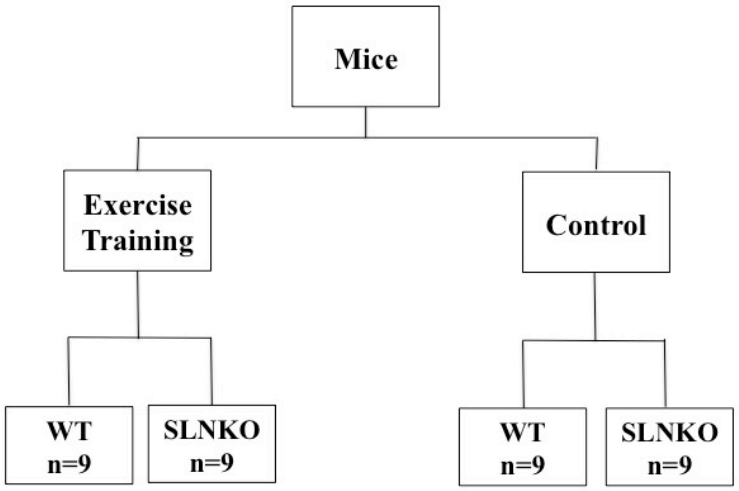

Figure 3. Experimental design (WT: wild type; SLNKO: sarcolipin knock-out).

The exercise training protocol was similar to previous mouse exercise training studies (Leick *et al*., 2008; Narker et al., 2008; Silva et al., 2009; Safdar et al., 2011; McFarlan *et al*., 2012), but with minor modifications. Briefly, mice assigned to the exercise-training group ran 5 days per week for a total of 8 weeks, while those assigned to the control group walked at 9 m/min at 0° incline 3 times a week for 15 min to ensure control mice remained acclimated to the treadmill (**Figure 4**). The majority of exercise-trained mice completed the required amount of running time for each daily bout of exercise and completed the training protocol. Occasionally however, some exercise-trained mice were unable to maintain the training speed thus, were removed early during that exercise bout and did not complete the running time for that given day.

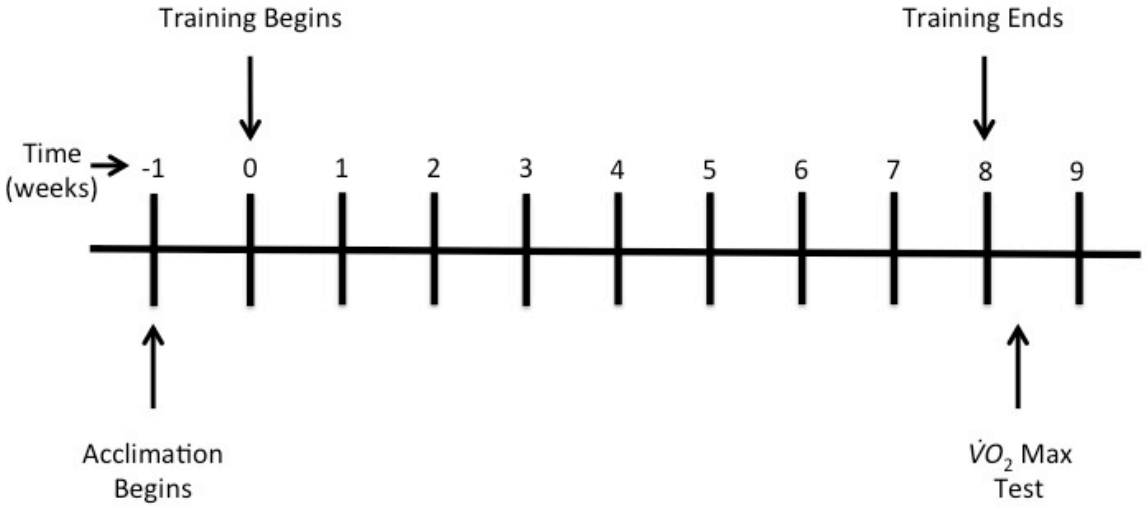

Figure 4. Experimental timeline for endurance exercise trained mice.

The exercise-training program was characterized by progressive increases in running speed and duration. Trained mice began each bout of exercise with a warm up starting at 8 m/min and the running speed was slowly increased by 1 m/min up to the target training speed for the week. The incline of the treadmill remained at 5° throughout the exercise program for trained mice. A final speed of 17 m/min and a running time of 60 min were achieved at the end of 8 weeks (**Table 1**).

Table 1. Endurance exercise training schedule

Week	Time (min)	Incline (°)	Speed (m/min)
1	15	5	13
2	30	5	13
3	45	5	13
4	60	5	13
5	60	5	14
6	60	5	15
7	60	5	16
8	60	5	17

$\dot{V}O_2$ Max Test

Maximal oxygen uptake ($\dot{V}O_2$ max) was measured in WT and SLNKO mice using an enclosed, airtight Modular Treadmill (Columbus Instruments, Columbus, OH), designed for integration with the Comprehensive Laboratory Animal Monitoring System (CLAMS; Oxymax Series, Columbus Instruments, Columbus, OH). Mice performed the $\dot{V}O_2$ max test two days after completing the exercise-training program.

The $\dot{V}O_2$ max test began with a 30 min sitting period within the enclosed treadmill which was set to a 5° incline. Measurement of $\dot{V}O_2$ began during the last 10 min of the sitting period. At the end of this period, mice began running at a speed of 3 m/min, after which the speed was increased incrementally by 3 m/min every 1.5 minutes (**Figure 5**). The test was terminated once the mouse was unable to maintain position on the treadmill (sat on the pulsed stimulus platform for approximately 15 s) and/or $\dot{V}O_2$ plateaued.

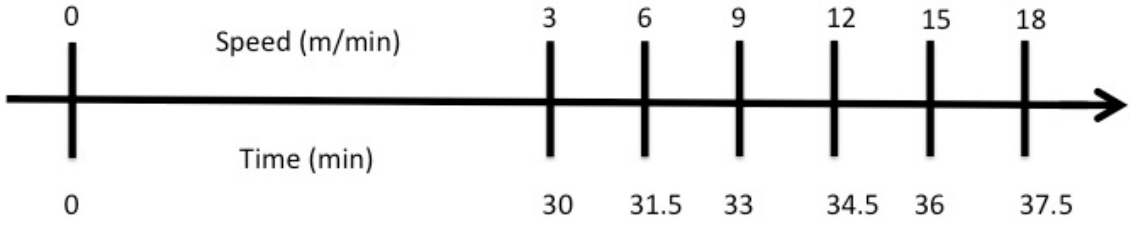

Figure 5. Experimental timeline for a $\dot{V}O_2$ max running test.

Tissue Collection

Two days following the $\dot{V}O_2$ max test, mice were fasted for 4 hours before tissue was collected. Mice were euthanized by cervical dislocation and weighed. The soleus and extensor digitorum longus (EDL) muscles of both hind limbs were immediately excised, dissected free of any connective tissue or visible fat and weighed. One set of skeletal muscles were frozen immediately in liquid N_2 and stored at -80ºC until further enzymatic

analysis (described below). Skeletal muscle from the contralateral limb were diluted 10:1 (vol/wt) in ice cold PMSF buffer (250 mM sucrose, 5 mM HEPES, 10 mM NaN_3, and 0.2 mM phenylmethanesulfonyl fluoride, pH 7.5) and homogenized using a handheld glass homogenizer before being frozen immediately in liquid nitrogen at -80ºC. These homogenates were used to examine the tissue expression of mitochondrial proteins by Western blotting analysis. The total protein concentration of all samples was determined in triplicate using the bicinchoninic acid (BCA) procedure using bovine serum albumin (BSA) standards (Sigma-Aldrich) and BCA assay reagents.

SDS-Page and Western Blotting

Relative protein expression of adenine nucleotide translocase (ANT), cytochrome-*c* (cyt-c) and cytochrome c oxidase subunit IV (COXIV) was determined in homogenates of both soleus and EDL muscles. Proteins of interest were separated by size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Equal quantities of proteins were loaded into each well while densities of polyacrylamide gels (Mini-PROTEAN II, Bio-Rad, Canada) varied as a result of differences in size between proteins measured (14% for ANT and cyt-c; 12.5% for COXIV). Following separation, proteins were transferred to a polyvinylidene difluoride membrane (PVDF membrane; Bio-Rad, Canada) using a semi-dry transfer unit at 23 mV for 45 min (Trans-Blot Cell, Bio-Rad, Canada). Membranes were then blocked for one hour with 5% skim milk in Tris-buffered saline (pH 7.5) at room temperature followed by incubation with primary anti-ANT1/2 (N-19) (1:100; sc-9299), anti-cytochrome-*c* (A-8) (1:2000; sc-13156) or anti-COX subunit IV (1:5000; mAbcam). Afterwards, membranes were washed with

Tris-buffered saline 0.1% Tween followed by treatment with the appropriate horseradish peroxidase-conjugated anti-mouse or anti-goat secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed a final time and signals were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ) using a bio-imaging system and densitometric analysis using GeneSnap software (Syngene, Frederick, MD). All membranes were stained with Ponceau S to confirm equal loading and allowed for normalization of densitometric results.

Enzyme Assays

Enzymes chosen for analysis that represented their respective metabolic pathways included citrate synthase (CS) and succinate dehydrogenase (SDH) of the citric acid cycle, cytochrome c oxidase (COX) of the ETC, and β-hydroxyacyl-CoA dehydrogenase (β-HAD) of mitochondrial fatty-acid oxidation. Muscle samples were homogenized 1:50 (w/v) in ice-cold phosphate-glycerol buffer (containing 16 mM Na_2HPO_4, 4 mM KH_2PO_4, 0.02% BSA, 5 mM 2-mercaptoethanol, and 0.5 mM EDTA) using a glass homogenizer and stored at -80ºC. Maximal activities of all enzymes were measured in triplicate, except COX, using NAD^+/NADH-linked fluorometric end-point assays at room temperature (~22ºC). To avoid repeated freezing/thawing, SDH was measured on freshly homogenized tissue while all other enzymes were measured on thawed homogenate. Assay procedures for all enzymes except for COX were carried out according to Henriksson and colleagues (1986), as modified by Green and colleagues (1995).

COX activity was measured using a reaction mixture containing 970 µl of 10 mM potassium phosphate buffer and 20 µl of reduced cytochrome C (Sigma C-2506) at 37°C.

The original muscle homogenate was diluted 10:1 using 10 mM potassium phosphate buffer to produce a final dilution of 1:500 of the original homogenate. The reaction was run in duplicate and began with adding 10 µl of dilute homogenate to the reaction mixture. Decreases in absorbance at 550 nm was measured spectrophotometrically for 3 min. COX activity was calculated using the measured slope and millimolar extinction coefficient of reduced cytochrome C at 550 nm, and expressed as millimoles per hour per gram protein.

Statistical Analysis

All data were analyzed using a two-way analysis of variance (ANOVA). When appropriate, post-hoc comparisons were made using a Newmans-Keuls test to examine specific mean differences. If interaction trends ($P<0.1$) were observed then student t-tests were employed for additional post-hoc analysis. Data are presented as mean ± standard error (S.E.). Statistical significance was considered at $\alpha = 0.05$.

Results

$\dot{V}O_2$ Max and Body Weights

The final body weights (g) of control (WT, 37.9 ± 1.3; SLNKO, 35.7 ± 0.7) and trained (WT, 38.7 ± 1.2; SLNKO, 36.8 ± 0.8) WT and SLNKO mice were not different and were unchanged from the initial weight of control (WT, 37.4 ± 1.1; SLNKO, 36.4 ± 0.9) and trained (WT, 40.0 ± 1.7; SLNKO, 38.5 ± 0.8) counterparts. The $\dot{V}O_2$ max values of control and trained mice are displayed in **Figure 6**. Exercise training did not significantly increase $\dot{V}O_2$ max for WT (5569 ± 185 ml/kg/hr vs. 5533 ± 169 ml/kg/hr) or SLNKO (6069 ± 162 ml/kg/hr vs. 5952 ± 274 ml/kg/hr) mice. SLNKO mice had greater relative $\dot{V}O_2$ max values compared to WT mice (P=0.05) regardless of training status (6030 ± 194 ml/kg/hr vs. 5551 ± 171 ml/kg/hr, respectively); however, absolute $\dot{V}O_2$ max did not significantly differ between WT mice and SLNKO mice (3.53 ± 0.3 l/min vs. 3.58 ± 0.3 l/min, respectively). In addition to relative $\dot{V}O_2$ max, SLNKO mice achieved a significantly greater running speed during $\dot{V}O_2$ max testing compared to WT mice (P<0.0001) regardless of training status (40 ± 1.0 m/min vs. 33 ± 1.3 m/min respectively).

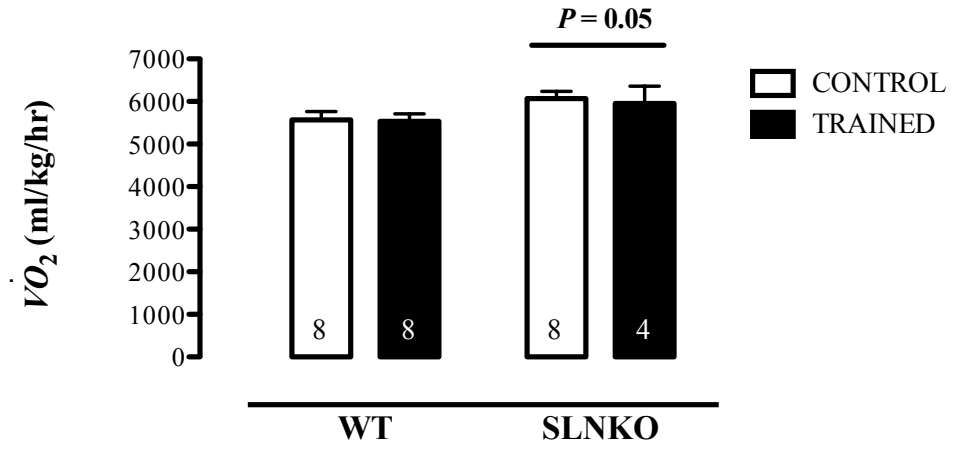

Figure 6. Comparison of $\dot{V}O_2$ max (ml/kg/hr) in control wild type (WT; n=8) and sarcolipin knockout (SLNKO; n=9) mice to trained WT (n=8) and SLNKO (n=4) mice. SLNKO mice had higher $\dot{V}O_2$ max than WT mice, P=0.05. Values are presented as mean ± S.E.

Expression of Mitochondrial Proteins

To assess mitochondrial biogenesis, the relative expression of a select number of mitochondrial proteins was measured. A main effect of exercise training was observed that was independent of genotype for both soleus and EDL. Therefore WT and SLNKO mouse data were group together and comparisons between control and trained animals were performed. Exercise training significantly increased ANT (P<0.03), cyt-*c* (P<0.02) and COXIV (P<0.03) expression by 14%, 33% and 28%, respectively, in the soleus independent of genotype (**Figure 7**). Similarly in EDL, exercise training induced significant increases in cyt-*c* (P<0.04) and COXIV (P<0.01) by 31% and 26%, respectively, but increases in ANT expression (19%) were insignificant (P=0.13; **Figure 8**).

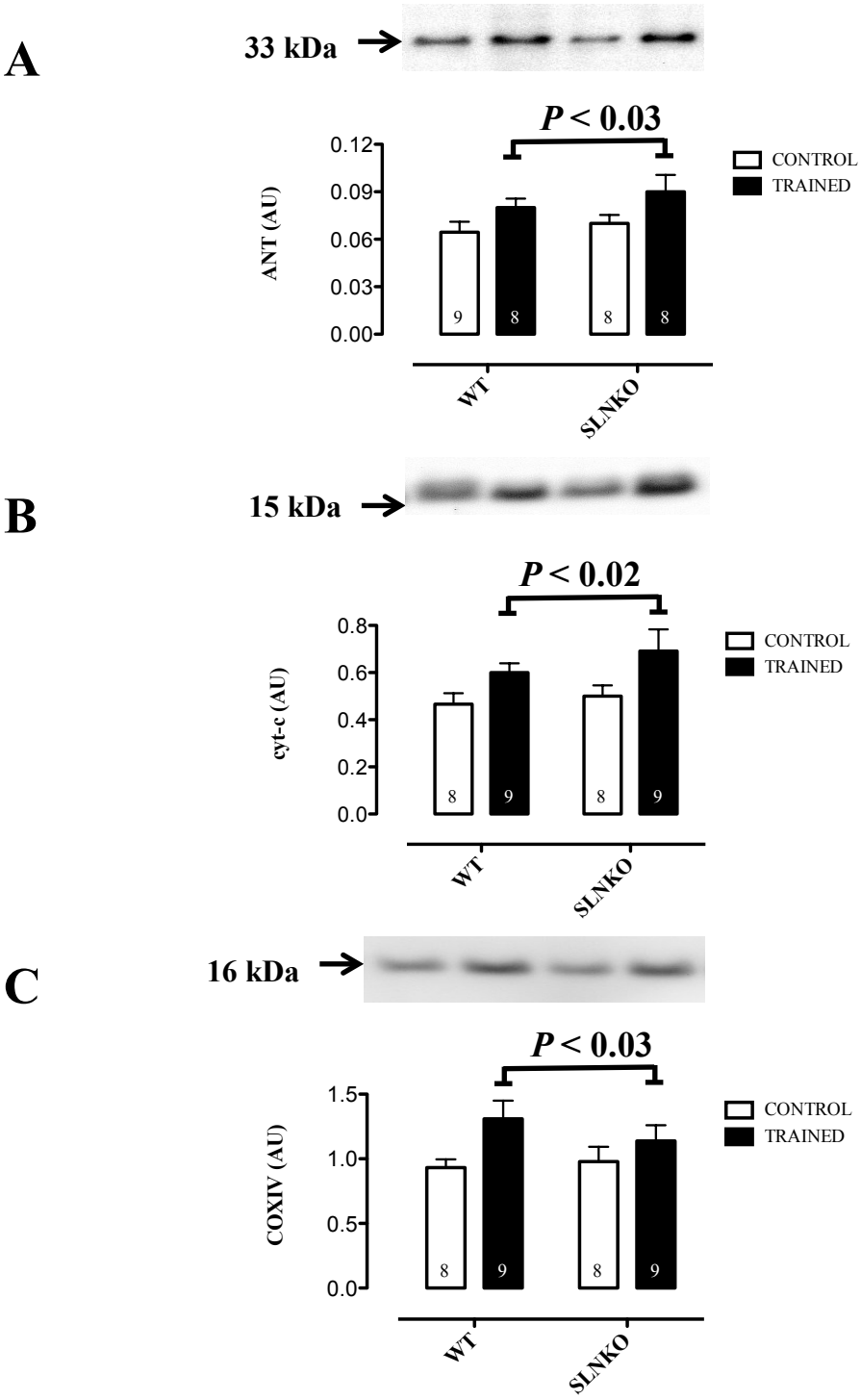

Figure 7. Relative expression of mitochondrial proteins in soleus of wild type and sarcolipin knockout mice in control and trained groups. Proteins are expressed in arbitrary units (AU). **A)** adenine nucleotide translocase (ANT). **B)** cytochrome-*c* (cyt-*c*). **C)** cytochrome *c* oxidase subunit IV (COXIV). A significant main effect of exercise training was found (trained > control) independent of genotype ($P<0.05$) for all proteins. Sample sizes for each group are indicated at the bottom of each bar. Values are mean ± S.E.

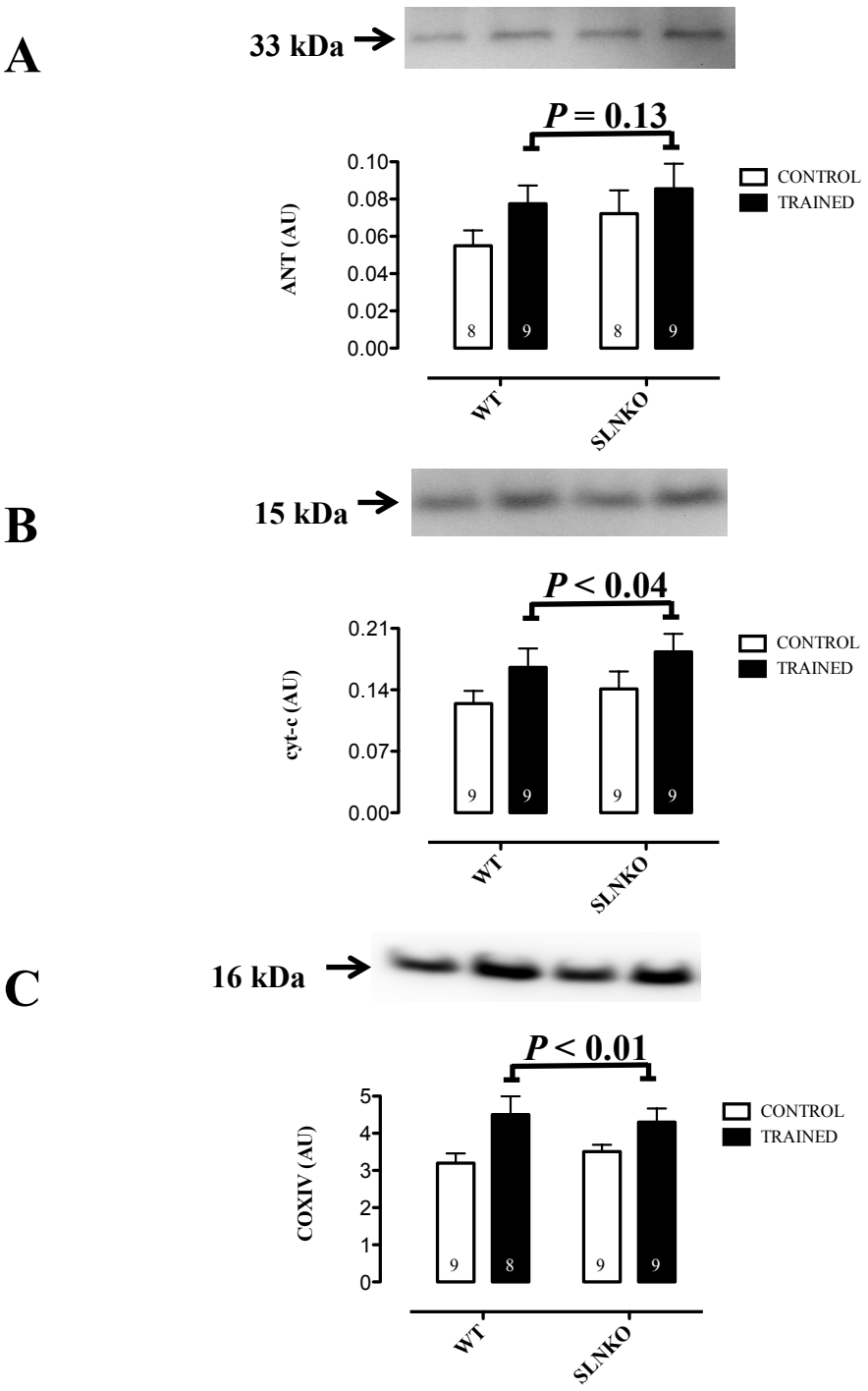

Figure 8. Relative expression of mitochondrial proteins in extensor digitorum longus of wild type and sarcolipin knockout mice in control and trained groups. Proteins are expressed in arbitrary units (AU). **A)** adenine nucleotide translocase (ANT). **B)** cytochrome-*c* (cyt-*c*). **C)** cytochrome *c* oxidase subunit IV (COXIV). A significant main effect of exercise training existed (trained > control) independent of genotype ($P<0.05$) for cyt-*c* and COXIV. Exercise training did not significantly increase ANT ($P=0.13$). Sample sizes for each group are indicated at the bottom of each bar. Values are mean ± S.E.

Skeletal Muscle Mitochondrial Enzyme Activities

To determine if skeletal muscle oxidative capacity was increased following training, the maximal activities of SDH, β-HAD, CS, and COX were measured. Two-way ANOVA results revealed that a genotype-training interaction existed for SDH; specifically, exercise training increased SDH activity in SLNKO (26%) but not in WT soleus (**Figure 9A**; Interaction effect, $P<0.02$). In contrast to SDH, exercise training significantly increased COX ($P=0.01$; **Figure 9B**) and CS ($P<0.01$; **Figure 9C**) enzyme activities in soleus by 11% and 17%, respectively, independent of genotype. Post-hoc comparisons indicated that genotype-training interactions were not significant ($P=0.06$) for COX and CS activities, however comparisons with student's t-tests revealed that exercise training significantly elevated COX ($P<0.02$) and CS ($P<0.01$) activity only in SLNKO and not WT soleus (**Table 2**). Two-way ANOVA results showed exercise training tended ($P=0.08$) to increase β-HAD activity in soleus (**Figure 9D**) and comparisons with student's t-tests revealed no differences between genotypes (**Table 2**).

Within the EDL, exercise training only increased SDH activity (**Figure 10A**; $P<0.05$) by 16%, with no significant differences between genotype. Exercise training did not significantly alter activities of COX, CS or β-HAD in the EDL (**Figure 10C-D**).

Table 2. Mitochondrial enzyme activities of control or trained wild type and sarcolipin knockout mice in the soleus. SDH: succinate dehydrogenase, β-HAD: β-hydroxyacyl-CoA dehydrogenase, CS: citrate synthase, COX: cytochrome *c* oxidase. Activities are expressed as mean ± S.E. (n) and in units mmol/hr/g prot. *Activity following exercise training was significantly different ($P<0.02$) than control group as determined by student's t-test.

	Control		Trained	
Enzyme	**WT**	**KO**	**WT**	**KO**
SDH	4.20 ± 0.27 (8)	3.61 ± 0.09 (8)	4.15 ± 0.19 (9)	4.55 ± 0.11* (8)
COX	1.73 ± 0.05 (8)	1.76 ± 0.09 (9)	1.94 ± 0.15 (9)	2.15 ± 0.11* (9)
CS	39.3 ± 1.28 (3)	37.19 ± 0.40 (3)	42.23 ± 1.06 (6)	42.93 ± 0.85* (6)
β-HAD	8.15 ± 0.60 (8)	7.29 ± 0.52 (8)	8.77 ± 0.63 (9)	8.40 ± 0.57 (9)

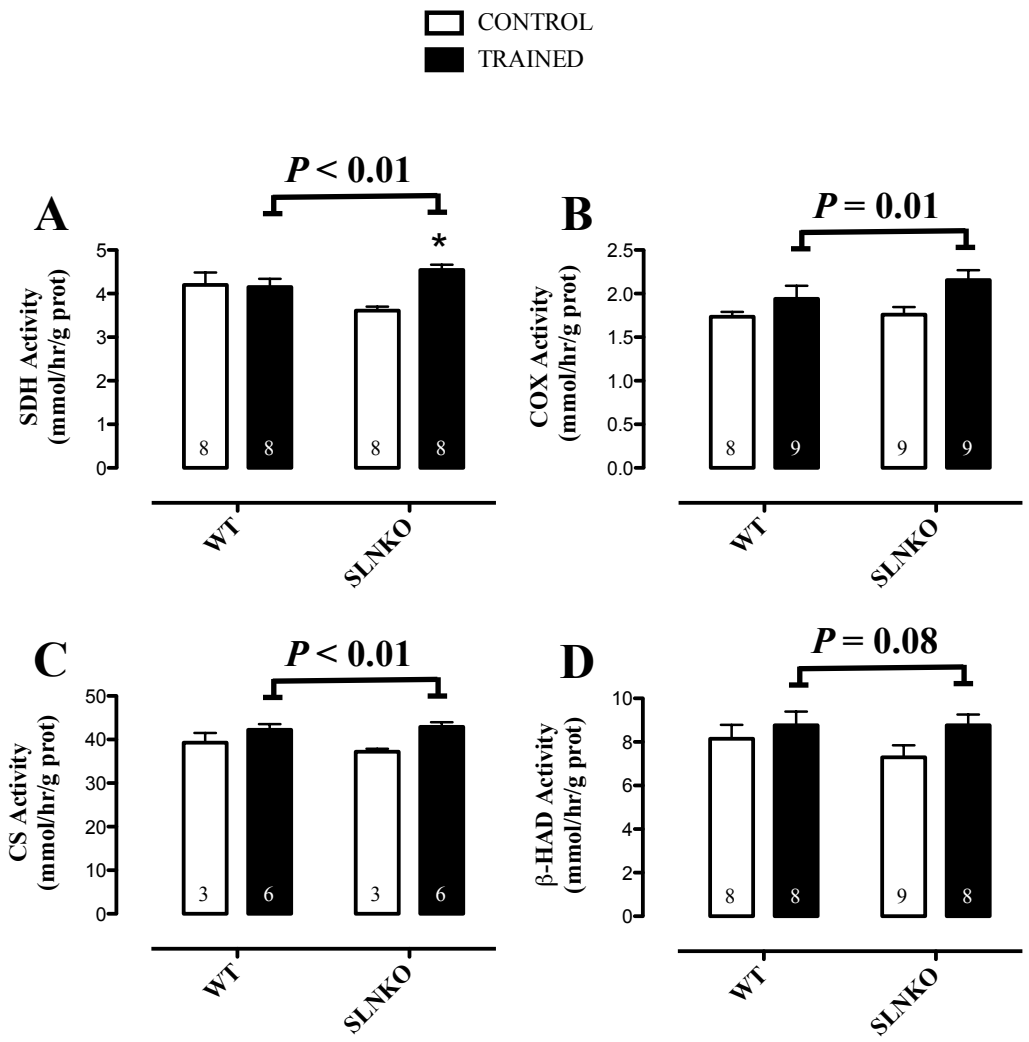

Figure 9. Mitochondrial enzyme activities within the soleus of wild type and sarcolipin knockout mice in control or trained groups. All enzymes activities are expressed in mmol/hr/g prot. **A)** succinate dehydrogenase (SDH) activity. **B)** cytochrome *c* oxidase (COX) activity. **C)** citrate synthase (CS) activity. **D)** β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity. A significant main effect of exercise training existed (trained > control) independent of genotype ($P<0.05$) for CS and COX while a trend towards increased β-HAD activity ($P=0.08$) was observed. Sample sizes for each group are indicated at the bottom of each bar. * Interaction observed where SDH activity following exercise training was significantly greater than control SLNKO mice ($P<0.02$). Values are mean ± S.E.

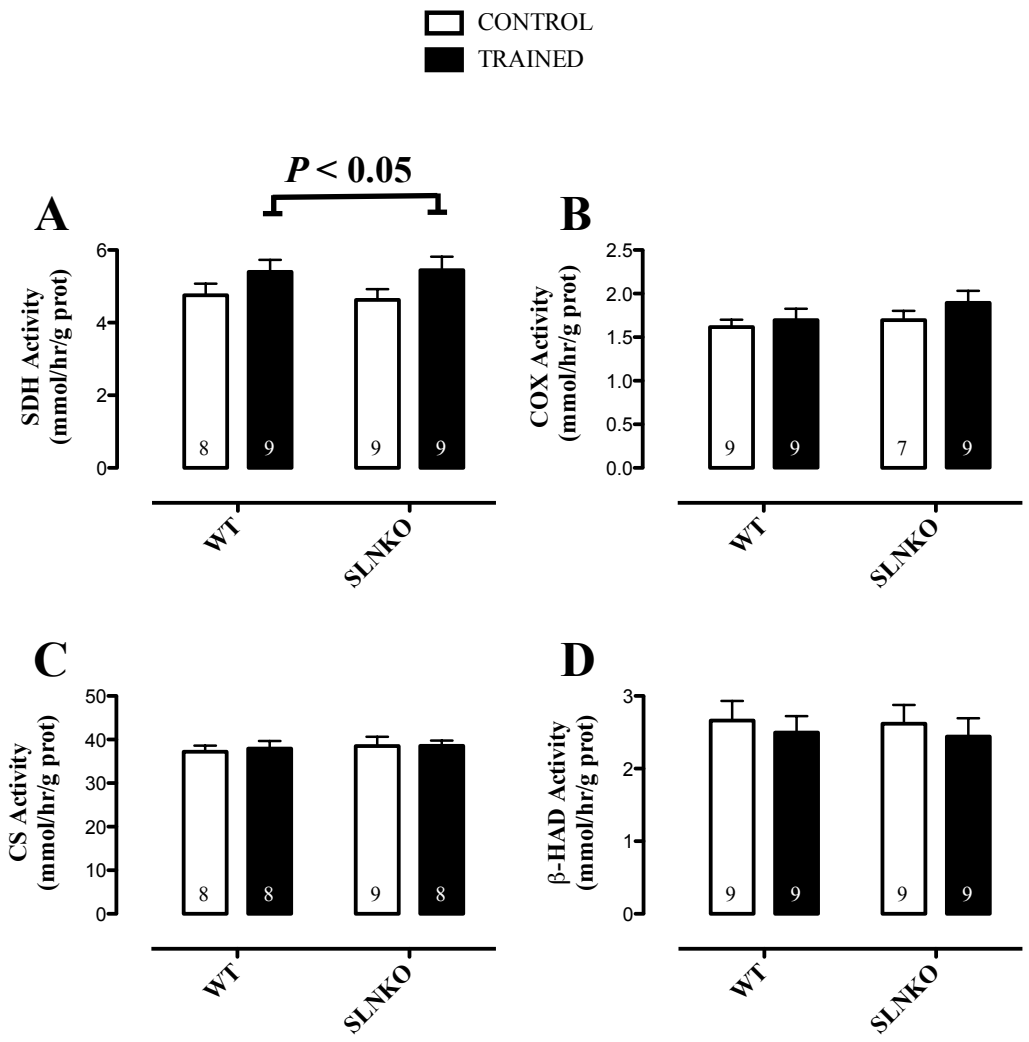

Figure 10. Mitochondrial enzyme activities within the extensor digitorum longus of wild type and sarcolipin knockout mice in control or trained groups. All enzymes activities are expressed in mmol/hr/g prot. **A)** succinate dehydrogenase (SDH) activity. **B)** cytochrome *c* oxidase (COX) activity. **C)** citrate synthase (CS) activity. **D)** β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity. A significant main effect of exercise training existed (trained > control) independent of genotype ($P<0.05$) for SDH. Exercise training did not alter activities of any other enzymes. Sample sizes for each group are indicated at the bottom of each bar. Values are mean ± S.E.

Discussion

Prior work by Gamu (2012) showed that skeletal muscle oxidative metabolism did not differ between WT and SLNKO mice under sedentary or voluntary exercising conditions. In that study, voluntary wheel running may have been too mild to induce an adaptive response and reveal potential phenotype differences. Therefore this thesis expanded on the previous study by using a forced exercise training protocol to investigate if increases in mitochondrial content in soleus and EDL muscles differed between WT and SLNKO in response to exercise training. For soleus where SLN is abundant, it was expected that ablation of SLN would reduce the signals inducing increases in mitochondrial content (i.e. increased $[Ca^{2+}]_f$ and AMPK activation) leading to attenuation of the adaptive response. In contrast, no genotype differences following exercise training were expected in EDL due to low endogenous SLN expression in that muscle. Exercise training has been well established as an inducer of mitochondrial biogenesis (Holloszy, 1967; Chow *et al*., 2007). Although $\dot{V}O_2$ max was not significantly increased following training (**Figure 6**), skeletal muscle oxidative capacity of trained mice was increased. The expression of mitochondrial proteins was significantly greater in both soleus and EDL independent of genotype (**Figure 7 and 8**). In soleus, exercise training elevated SDH activity only in SLNKO mice (**Figure 9A**) whereas other mitochondrial enzyme activities were increased independent of genotype (**Figure 9**). Post-hoc comparisons indicated that genotype-training interactions were not significant (P=0.06) however, further comparisons using student t-tests revealed that COX and CS activities were significantly increased only in SLNKO mice and not WT controls, consistent with SDH activity results. In the EDL, exercise training only increased SDH activity whereas other

mitochondrial enzymes remained unaltered (**Figure 10**). Overall, the results of this study do not support the original hypothesis since SLN ablation does not attenuate the adaptive increase in mitochondrial content in response to exercise training. Instead, this study provides some evidence that SLN ablation may increase the sensitivity of skeletal muscle to exercise training and augment mitochondrial enzyme activities. Future investigations are needed to confirm these results.

Increases in $\dot{V}O_2$ max have been demonstrated in mice under voluntary wheel running (Swallow *et al*., 1998) or forced exercise-training conditions (Kemi *et al*., 2002; Chow *et al*., 2007). The lack of improvement in $\dot{V}O_2$ max of trained mice (**Figure 6)** comes surprisingly considering mitochondrial protein expression and enzyme activities (**Figure 7-10**) were increased in trained mice. Underlying this divergence in results is the potential contribution of mouse strain on performance during treadmill running. Compared to other strains, C57BL/6J mice are the poorest performers during forced endurance exercise testing (Lerman *et al*., 2002) and display lower aerobic capacities as assessed by graded treadmill testing (Lightfoot *et al*., 2001). It has been shown that high intra-mouse variability exists for repeated treadmill testing with an enclosed chamber and shock stimulus (Knab *et al*., 2009) thus, lack of improvement in $\dot{V}O_2$ max could reflect C57BL/6J mice running performances during treadmill stress testing and not due to the lack of adaptive changes. Previous aforementioned exercise studies from which this exercise training protocol was modeled used time-to-exhaustion testing to determine genotype or training differences (Narker *et al*., 2008; Safdar *et al*., 2011; McFarlan *et al*, 2012), which may have been a more appropriate test to assess functional differences. Interestingly, SLNKO mice displayed greater relative $\dot{V}O_2$ max values compared to WT

mice regardless of training status, in contrast to previous observations (Bombardier *et al*., unpublished). Absolute $\dot{V}O_2$ max did not significantly differ between genotypes, indicating the slightly lower weights of SLNKO mice likely contributed to the higher $\dot{V}O_2$ max values. Interestingly, SLNKO mice achieved significantly greater running speeds compared to WT mice during $\dot{V}O_2$ max testing (P<0.0001; 40 ± 1.0 m/min vs. 33 ± 1.3 m/min respectively), suggesting greater running performance in SLNKO mice compared with WT.

In addition to $\dot{V}O_2$ max, increases in mitochondrial protein content and enzyme activities in skeletal muscle are hallmark characteristics of exercise training (Holloszy and Booth, 1976). Observed increases in cyt-*c* and COXIV, electron transport chain (ETC) proteins, and ANT, a key regulator of mitochondrial ATP/ADP flux, would indicate greater mitochondrial size and/or number in both soleus and EDL. However, ANT expression was significantly increased in soleus (**Figure 7A**) but not in EDL (**Figure 8A**). Previous exercise training studies have demonstrated skeletal muscle increases of cyt-*c* and COXIV expression in mice (Leick *et al*., 2008; Li *et al*., 2011; McFarlan *et al*., 2012), and ANT expression in humans (Fernström *et al*., 2004). Unexpectedly, the increases in mitochondrial proteins with training were independent of genotype, indicating that SLNKO mice do not display an attenuated adaptive response to exercise training compared with WT mice.

Coinciding with protein expression, exercise training would be expected to elevate maximal activities of mitochondrial enzymes involved in the ETC (SDH and COX), citric acid cycle (CS), and fatty acid oxidation (β-HAD). Increases in the aforementioned enzymes are a well-characterized response to long-term running and has

been observed in a number of mouse exercise studies (Davidson *et al*., 2003; Chow *et al*., 2007; McFarlan *et al*., 2012). Two-way ANOVAs, indicated that with the exception of SDH, increases in COX, CS and β-HAD activities within the soleus (**Figure 9**) following exercise training occurred independent of genotype. A genotype-training interaction for SDH was observed where exercise training increased SDH activity within the soleus of SLNKO mice but not in WT mice, which was opposite to what was hypothesized and not totally consistent with the protein expression data. Genotype-training interactions for COX and CS were not significant (P=0.06); however detection of interactions may have been limited by small sample sizes. Comparison by student's t-tests were conducted and it was revealed that significant increases in COX and CS activity were confined to trained SLNKO mice, similar to SDH activity. These results indicate that SLN ablation did not attenuate the adaptive response to exercise training as hypothesized but instead, may have increased mitochondrial enzyme adaptations. In contrast to the soleus, exercise training increased only SDH activity in EDL, an effect that was independent of genotype and consistent with the hypothesis, and all other measured enzymes were unaltered (**Figure 10**). It is unclear whether the increases in mitochondrial enzyme activities reflect greater protein expression or indicate that activation of enzymes is greater thus, potentially contributing to the discrepancy seen between mitochondrial protein expression and enzyme activities in relation to genotype.

Due to the endurance nature of the exercise program, activation of Ca^{2+} and/or AMPK mediated signaling likely resulted in greater PGC-1α activity and expression, subsequently leading to mitochondrial biogenesis. Greater increases in mitochondrial enzyme activities in SLNKO mice indicate that SLN ablation might augment the adaptive

response to exercise training. Historically, SR Ca^{2+} release has been implicated as the major source of Ca^{2+} in skeletal muscle contraction (Melzer *et al*., 1995) and signaling for gene expression (Chin, 2005). More recently, there is growing evidence indicating that additional sources of Ca^{2+} may be important contributors to contraction and mediate signaling in skeletal muscle (Dirksen, 2009; Rosenberg *et al*., 2009). Specifically, store operated Ca^{2+} entry (SOCE) has been suggested to be a requirement in replenishing and sustaining SR [Ca^{2+}] required for contraction. Depletion of SR Ca^{2+} is sensed by stromal interaction molecule 1 (STIM1), which induces a conformational change and translocation of the protein to the SR-plasma membrane junction. Here, it activates the highly selective Ca^{2+} channel Orai1 via protein-protein interaction and allows entry of extracellular Ca^{2+} into the SR and cytosol (Dirksen, 2009; Soboloff *et al*., 2012). Myocytes devoid of STIM1 display diminished SR [Ca^{2+}] content (Seth *et al*., 2012) and during repetitive stimulation, subsequent Ca^{2+} transient amplitudes are rapidly decreased (Stiber *et al*., 2008). Thus, refilling of the SR stores via SOCE is important for maintaining Ca^{2+} release required in generating contractile force during exercise.

Additionally, SOCE has been shown to modulate Ca^{2+} transients and influence Ca^{2+}-mediated transcriptional signaling (Stiber *et al*., 2011). Recent evidence has demonstrated that the overexpression of activated STIM1 in myotubes increases NFAT activation (Stiber *et al*., 2008), which has been demonstrated to be important in skeletal muscle adaptation to exercise (Chin *et al*., 1998; Jiang *et al*., 2010). Moreover, protein expression of NFAT and transcription factors PGC-1α, PPARs β/δ and MEF2 were reduced in skeletal muscle of STIM1-*null* mice compared to WT animals (Li *et al*., 2012). STIM1 ablation reduces SOCE causing decreased Ca^{2+} signaling that would

contribute to the reduction of transcription factors regulating mitochondrial biogenesis. Interestingly during muscle development, STIM1-*null* myotubes exhibit marked increases in SLN expression (Seth *et al*., 2012). In skeletal muscle, SLN overexpression was shown to reduce SR [Ca^{2+}] load and impair contractility (Tupling *et al*., 2002) while SLN ablation in atrial myocytes resulted in greater SR [Ca^{2+}] stores and enhanced contractility (Babu *et al*., 2007). Likewise, overexpression of SLN in myotubes reduced STIM1 expression, reduced SOCE and delayed muscle differentiation indicating SLN and STIM1 are expressed in opposing fashion (Seth *et al*., 2012). These current findings suggest that SLN may inhibit STIM1 and reduce SR [Ca^{2+}] content. Consequently, smaller SR [Ca^{2+}] stores results in less Ca^{2+} release, thereby attenuating Ca^{2+}-mediated transcriptional signaling. Collectively, these results suggest that SLN ablation could potentially activate or increase STIM1 expression leading to greater SOCE and SR Ca^{2+} stores which could possibly explain the results of this study, namely greater increases in mitochondrial enzyme activities in SLNKO mice following exercise training.

Recently, ablation of SLN in atrial myocytes was shown to increase SR Ca^{2+} leak through RyR, which was attributed to greater CaMKII activity, and resulted in atrial remodeling (Xie *et al*., 2012). Greater CaMKII activity corresponds to greater activation of Ca^{2+} signaling and increases mitochondrial biogenesis. By impairing SERCA function, SLN blocks refilling of SR Ca^{2+} and STIM1 action and possibly attenuate Ca^{2+} signaling. Ablation of SLN could potentially increase SR [Ca^{2+}] stores which subsequently gives rise to greater $[Ca^{2+}]_f$ amplitudes and increased activation of CaMKII or calcineurin. As a result, the exercise induced adaptive response would be augmented in SLNKO mice, which is indicated by greater mitochondrial enzyme activity in the soleus (**Figure 9**) and

RG (**Appendix A**), which was used to confirm soleus results. In comparison, no significant genotype differences in mitochondrial protein expression or enzyme activities were found in the EDL (**Figure 10**), which is consistent with the fact that low endogenous levels of SLN are found in EDL naturally. The importance of SOCE in skeletal muscle contraction and signaling *in vivo* has only been recently investigated thus, future studies should determine the physiological relevance of SOCE in modulating SR function or cellular signaling, and whether SLN inhibits SOCE *in vivo*.

Inherent differences in adaptive capacity exist between subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria, with SS mitochondria displaying greater metabolic plasticity to exercise (Krieger *et al*., 1980; Bizeau *et al*., 1998; Koves *et al*., 2005). Interestingly, mice expressing activated CaMKIV display a greater number of SS mitochondria (Wu *et al*., 2002). Moreover, exercise training (Koves *et al*., 2005) or modest PGC-1α overexpression (Benton *et al*., 2008) have been shown to enhance fatty acid oxidation in SS mitochondria compared to IM mitochondria. Thus, in response to a common stimulus (e.g. contractile activity), two distinct, compartmentalized signaling pathways mediating biogenesis of specific mitochondrial subpopulations may exist (Koves *et al*., 2005). Recent evidence of direct tethering of SS mitochondria to the SR (Boncompagni *et al*., 2009) suggests an interaction between mitochondria and SLN could potentially exist. Further research is warranted to determine if enhanced exercise training-induced mitochondrial biogenesis in SLNKO mice is specific to SS mitochondria.

Multiple signaling pathways mediate the adaptive response to exercise and there is a high degree of cross talk and redundancy among pathways. PGC-1α is regulated by Ca^{2+} (Wu *et al*., 2002; Lin *et al*., 2002; Handschin *et al*., 2003; Long *et al*., 2007;

Guerfali *et al*., 2007; Wright *et al*., 2007), AMPK (Irrcher *et al*., 2008; Cantó *et al*., 2009; Cantó *et al*., 2010;), p38 MAPK (Akimoto *et al*., 2005; Wright *et al*., 2007), and ROS (Kang *et al*., 2009), further supporting the complexity and convergence of exercise signals in mitochondrial biogenesis. Due to the prolonged nature of the exercise-training program, metabolic signals such as increased [AMP]/[ATP] ratio, would have induced AMPK activation and contributed to the adaptive response. These metabolic changes trigger the activation of AMPK, which has been shown to increase mitochondrial content in skeletal muscle (Winder *et al*., 2000; Irrcher *et al*., 2003; Koh *et al*., 2008). It would be expected that AMPK mediated signaling would be decreased in SLNKO mice given the fact that they display lower $\dot{V}O_2$ during submaximal exercising conditions (Bombardier *et al*., unpublished). Furthermore, the effect of SLN on skeletal muscle thermogenesis (Bombardier *et al*., unpublished; Bal *et al*., 2012) provides evidence that SLNKO mice are more energy efficient. Therefore, metabolic signaling would be expected to decrease in SLNKO mice relative to WT mice. Despite potential decreases in metabolic signaling, SLNKO mice displayed greater increases in mitochondrial enzyme activities compared to WT mice indicating the adaptive response was not attenuated and other signals, such as elevated $[Ca^{2+}]_f$, may be more important. In support of this notion, transgenic mice with impaired AMPK signaling display equal training induced increases in mitochondrial content (Jørgensen *et al*., 2007). This thesis did not measure the expression of signaling molecules such as AMPK or CaMKII, therefore limiting our conclusions to phenotype characteristics. Further investigation is needed to determine the relative importance of Ca^{2+} and/or AMPK mediated signaling in skeletal muscle of SLNKO mice.

The extent of an adaptive response generally reflects the magnitude of the inducing stimulus. Although fast-glycolytic muscles, such as EDL, have a greater capacity to increase oxidative metabolism (Pette and Staron, 1997), these muscles are recruited less during endurance exercise compared to slow-oxidative muscles and consequently, undergo less of an adaptive response (Holloszy and Booth, 1976). Furthermore, the EDL is a non-weight bearing muscle that is activated during the swing phase of hind limb movement (Nicolopoulos-Stournaras and Iles, 1984), thus, the weight bearing stimulus of running would be reduced and attenuate the adaptive response. Moreover, buffering of Ca^{2+} by PV in fast twitch skeletal muscle could also lead to quicker $[Ca^{2+}]_f$ decay and potentially lower activation of calcineurin or CaMKIV. The lower recruitment and weight bearing stimulus imposed on EDL could contribute to the less robust increase in oxidative capacity observed in EDL, and dissociation between increased mitochondrial protein expression (**Figure 8**) and unaltered enzyme activities (**Figure 10**) following exercise training.

In summary, due to the key role SLN has in regulating Ca^{2+} handling and skeletal muscle thermogenesis, it was hypothesized that SLN ablation would attenuate the adaptive response to exercise training in slow-oxidative muscle. This thesis provides evidence that ablation of SLN does not attenuate the adaptive response of soleus muscle to exercise training. Furthermore, fast-twitch muscles were not expected to display genotype differences due to low endogenous levels of SLN which is supported by EDL, which exhibited no genotype differences in mitochondrial markers that were increased with exercise training. There is some evidence suggesting the adaptive response may be greater in SLNKO mice, demonstrated by increases in mitochondrial enzyme activities

following exercise training in the soleus of SLNKO mice. Future investigations should be conducted to confirm these findings. The effects of SLN ablation specifically on Ca^{2+} and AMPK mediated signaling in mitochondrial biogenesis remains to be determined and the physiological mechanisms regulating SLN function in skeletal muscle are poorly understood thus, future studies should also address these problems to further elucidate the role of SLN in exercise training.

Study Limitations

The greatest limitation of this study was the difficulty of getting mice to exercise. Mice often refused to run despite having previous treadmill exposure with the use of shock or physical stimuli, making it highly difficult to implement exercise training. It has been noted that up to 25% of rats refuse to run on a treadmill (Bedford *et al*., 1979; Kregel *et al*., 2006) although no observations to the author's knowledge have been made for mice. Moreover, the SLNKO mice were crossbred on a C57BL/6J background, a strain of mice that are among the worst performers on a treadmill compared to other mice (Lightfoot *et al*., 2001; Lerman *et al*., 2002). Furthermore, due to high variability that exists for repeated treadmill running (Knab *et al*., 2009), it is difficult to ensure that each bout of exercise is equal in running performance and exertion for individual mice, potentially affecting the adaptive response.

Due to the high degree of cross talk and redundancy among signaling pathways in exercise induced mitochondrial biogenesis, the alteration of a specific pathway during exercise may be masked by another pathway to maintain the adaptive response. For example, AMPKα2-null mice display normal training induced increases in mitochondrial enzymes despite having impaired AMPK signaling (Jørgensen *et al*., 2007). The lack of AMPK signaling was likely mitigated by Ca^{2+} signaling, thereby demonstrating redundancy in signaling pathways to maintain a normal adaptive response. Although SLNKO mice exhibited greater mitochondrial content following exercise training, changes to the underlying mechanisms or signaling were not determined in this thesis thus, restricting the conclusion to phenotype changes. Measuring the expression of signaling molecules such as CaMKII and AMPK would provide greater information

regarding the effects of SLN ablation specifically on Ca^{2+} and/or metabolic signaling. Additionally, the emerging importance of SOCE to skeletal muscle function and signaling suggests the expression of STIM1 and Orai1 should have been examined in response to SLN ablation and exercise. This would have given greater insight into the potential relationship between SLN and STIM1 in regulating $[Ca^{2+}]_f$.

Summary and Conclusions

In skeletal muscle, the increase in $[Ca^{2+}]_f$ or [AMP]/[ATP] resulting from exercise plays a significant role in mediating mitochondrial biogenesis. SLN has been established as a key regulator of Ca^{2+} handling and thermogenesis in skeletal muscle. Thus it was hypothesized that SLN ablation could influence Ca^{2+} and metabolic signaling thereby attenuating exercise induced mitochondrial biogenesis. Opposite to what was hypothesized, this study demonstrated that the phenotypic adaptive response to exercise training in skeletal muscle was not attenuated within the soleus of SLNKO mice. $\dot{V}O_2$ max was found to be unchanged with exercise training in both genotypes however mitochondrial protein expression was significantly increased in response to training, independent of genotype. Unlike mitochondrial protein expression, mitochondrial enzyme adaptations appeared to be greater in SLNKO mice, which displayed significantly greater SDH, COX and CS activities within the soleus following exercise training. In contrast to soleus, EDL displayed a less robust increase in mitochondrial content likely due to lower recruitment of the EDL during running. Moreover, the few mitochondrial markers that were increased with exercise training in EDL displayed no genotype differences, supporting the hypothesis that exercise-induced adaptations in muscles with low endogenous levels of SLN would be unaffected by genotype. It can be concluded that SLN ablation does not attenuate the adaptive response of skeletal muscle to exercise training but instead, may increase mitochondrial enzyme adaptations. Additionally, this thesis does not yield information regarding the effects of SLN ablation on Ca^{2+} or metabolic signaling, which should also be included in future investigations.

Future Directions

Inclusion of a time-to-exhaustion test at submaximal $\dot{V}O_2$ for post-exercise training assessments could be better reflective of aerobic exercise capacity, as a number of previous studies (Narker *et al*., 2008; Safdar *et al*., 2011; McFarlan *et al*, 2012) have used this test instead of $\dot{V}O_2$ max testing. The highly orchestrated network of signaling pathways involved in the exercise induced adaptive response indicates alterations to specific pathways should be determined to explain the mechanisms underlying the enhancement of mitochondrial biogenesis in SLNKO mice following exercise training. Biochemical and molecular analyses should include measurement of protein expression for AMPK, calcineurin, CaMKII and downstream targets such as NFAT, MEF2, and PGC-1α. Moreover, the emerging importance of SOCE to contractile function and signaling indicates the protein expression of STIM1 and Orai1 should be measured to determine if they are affected by exercise training in general. Furthermore, evidence showing SLN and STIM1 are expressed in opposing fashion during muscle differentiation suggests their relationship might influence Ca^{2+} signaling (Seth *et al*., 2012). Thus, further investigations should be made into examining possible interactions between SLN and SOCE in skeletal muscle of SLNKO mice. This will further elucidate the impact SLN has on Ca^{2+} signaling in skeletal muscle.

Increases in mitochondrial protein expression occurred independent of genotype whereas increases in mitochondrial enzyme activities appeared to be greater in the SLNKO mice. The greater mitochondrial enzyme activities could reflect post-translational modifications in the SLNKO mice that may differ from WT mice; however, given the length of the training period, increases could also indicate greater enzyme

protein expression. This cannot be determined from activity alone and measurement of mitochondrial enzyme protein expression should be included in future investigations to explain the discrepancy between protein expression and enzyme activities.

Additional measurements including morphological assessment and mitochondrial function should be included in future studies to yield more comprehensive results. Given the possibility that SS mitochondria are more sensitive to exercise training (Krieger *et al*., 1980), fractioning mitochondria into subpopulations and examining protein expression and oxidative enzyme activities would reveal population specific changes occurring with exercise. Furthermore, transmission electron microscopy would determine if morphological or proliferative changes of both SS and IM mitochondria are altered in response to SLN ablation. In addition to mitochondrial proliferation, exercise training is known to alter mitochondrial function and improve quality (Koves *et al*., 2005; Yan *et al*., 2012). Measurement of palmitate oxidation ([1-^{14}C]) or mitochondrial respiration ([state III]/[state IV]) for either whole muscle homogenates or isolated mitochondria would provide information pertaining to changes in mitochondrial function with exercise training. There are several sites in a metabolic pathway (i.e. uptake or catabolism) that could limit mitochondrial function, therefore a measurement such as palmitate oxidation would provide greater inference to functional alterations of mitochondria and allow for more comprehensive conclusions.

The physiological mechanisms regulating SLN function in skeletal muscle are poorly understood. Initially, studies demonstrated that SLN inhibition could be removed by β-adrenergic stimulation in cardiomyocytes (Asahi *et al*., 2004; Babu *et al*., 2006; Gramolini *et al*., 2006; Babu *et al*., 2007). It was also determined that CaMKII could

phosphorylate the threonine-5 (T5) residue of the N-terminus of SLN *in vitro* of cardiac myocytes (Bhupathy *et al*., 2009), providing a mechanism by which SLN function is modulated by phosphorylation. Unlike cardiac muscle, the physiological regulation of SLN in skeletal muscle has only recently been examined (Tupling *et al*., 2011, Bal *et al*., 2012; Bombardier *et al*., unpublished). Repeated tetanic stimulation at 70 Hz of mouse soleus was found to increase the rate of relaxation by the 10th tetanus in WT mice but not in SLNKO mice, suggesting SLN may have been inactivated (Tupling *et al*., 2011). Therefore, the onset of contractile activity would increase $[Ca^{2+}]_f$ thereby activating CaMKII and result in SLN phosphorylation at residue T5 to relieve inhibition and improve relaxation. Despite this evidence, recent studies have shown that SLN inhibition is not relieved during skeletal muscle contractile activity. Throughout submaximal exercise, WT mice display greater $\dot{V}O_2$ values compared to SLNKO mice (Bombardier *et al*., unpublished), indicating that SLN continues to uncouple ATP hydrolysis thereby increasing energy expenditure. Furthermore, cross-linking studies show SLN continues to interact with SERCA in the presence of high $[Ca^{2+}]_f$ (100 μM; Bal *et al*., 2012). Utilization of point mutations, measurement of SLN phosphorylation and SERCA Ca^{2+} uptake could yield information on the regulation of SLN function, and whether SLN inhibition is governed by phosphorylation/dephosphorylation processes in skeletal muscle.

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Appendix A

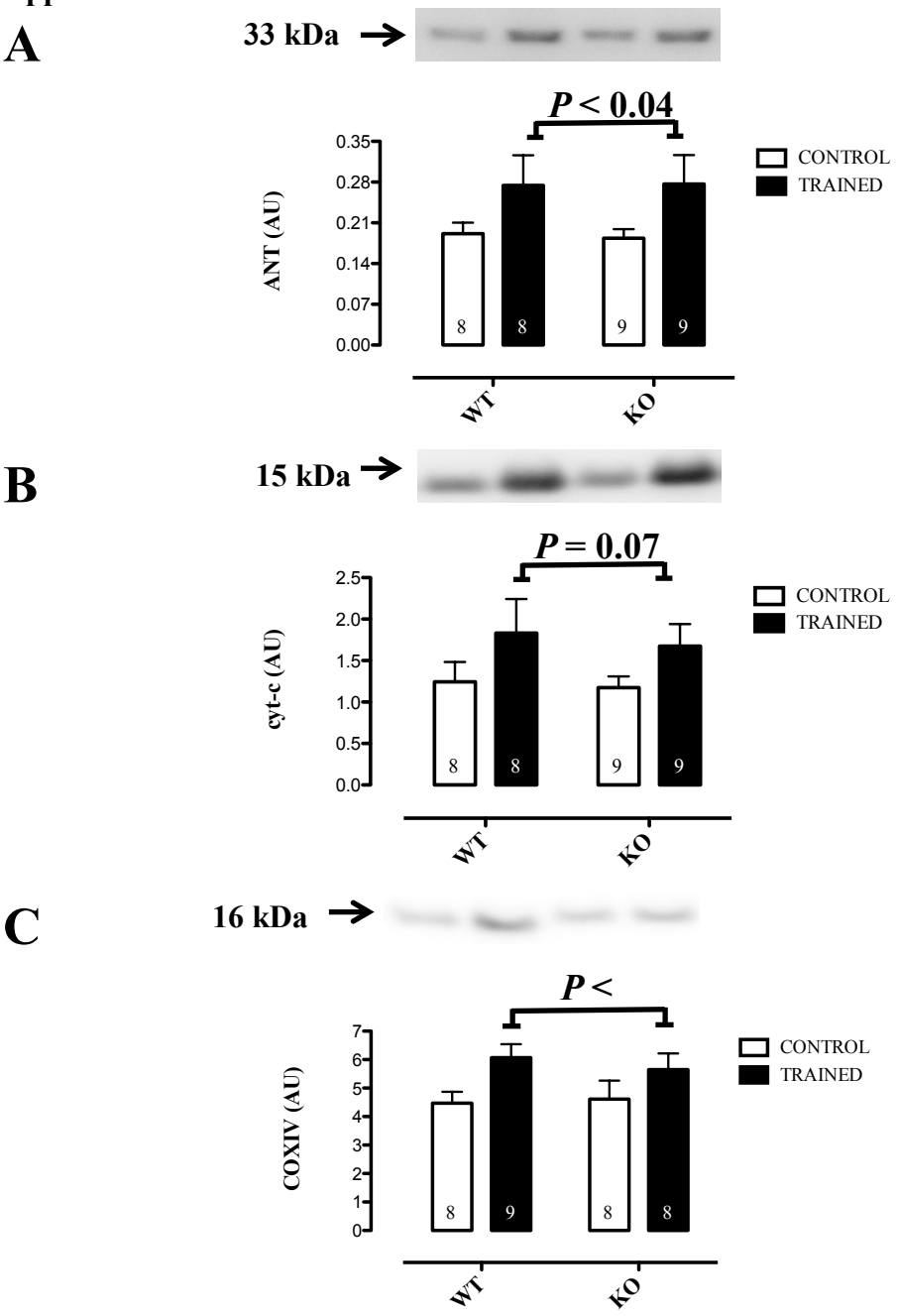

Figure A1. Relative expression of mitochondrial proteins in red gastrocnemius of wild type and sarcolipin knockout mice in control and trained groups. Proteins are expressed in arbitrary units (AU). **A)** adenine nucleotide translocase (ANT). **B)** cytochrome-*c* (cyt-*c*). **C)** cytochrome *c* oxidase subunit IV (COXIV). A significant main effect of exercise training existed (trained > untrained) independent of genotype ($P<0.05$) for ANT and COXIV. Exercise training did not significantly increase cyt-*c* ($P=0.07$). Sample sizes for each group are indicated at the bottom of each bar. Values are mean ± S.E.

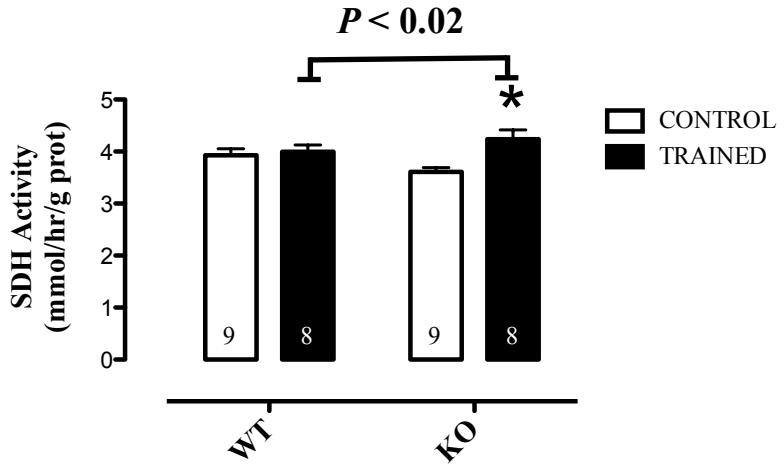

Figure A2. Succinate dehydrogenase activity (SDH) within the red gastrocnemius of wild type and sarcolipin knockout mice in control or trained groups. Enzyme activity is expressed in mmol/hr/g prot. Sample sizes for each group are indicated at the bottom of each bar. * Interaction observed where SDH activity following exercise training was significantly greater than control SLNKO mice ($P<0.04$). Values are mean ± S.E.

Appendix B

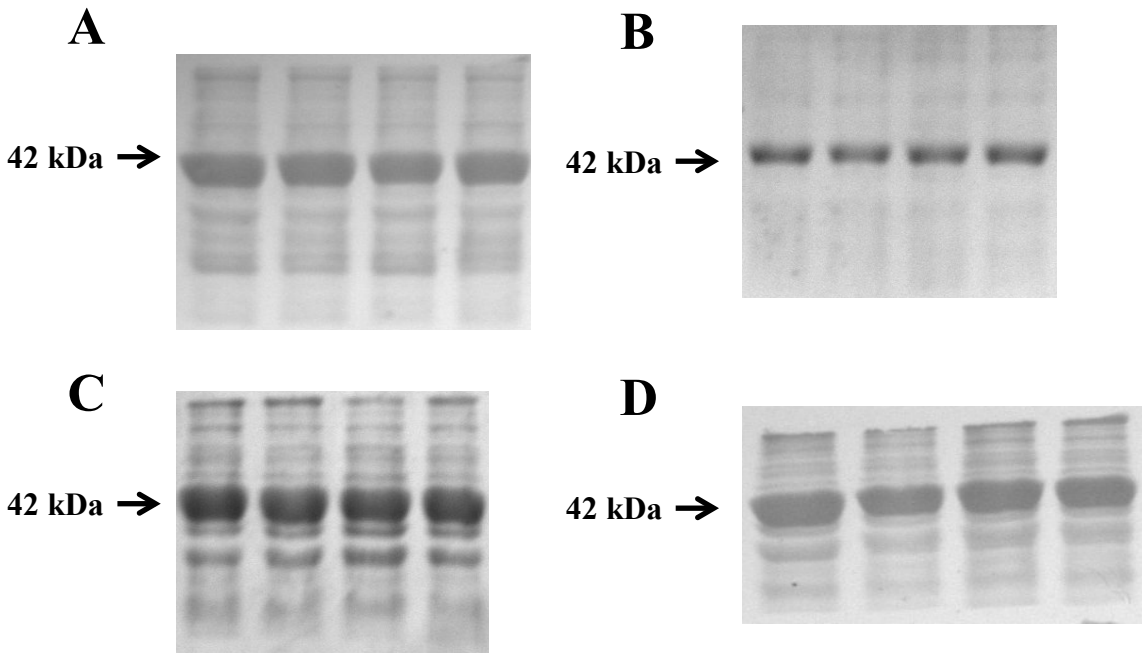

Figure B1. Nonspecific protein stains used to normalize adenine nucleotide translocase (ANT), cytochrome-*c* (cyt-*c*) and cytochrome *c* oxidase subunit IV (COXIV) expression in soleus and extensor digitorum longus (EDL). Actin is detected at ~ 42 kDa. Representative stains used for: **A)** cyt-*c* and ANT in soleus, **B)** COXIV in soleus, **C)** cyt-*c* and ANT in EDL, **D)** COXIV in EDL.

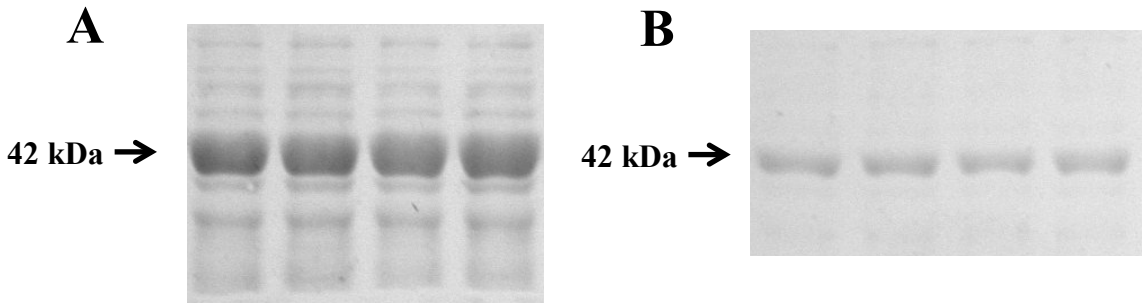

Figure B2. Nonspecific protein stains used to normalize adenine nucleotide translocase (ANT), cytochrome-*c* (cyt-*c*) and cytochrome *c* oxidase subunit IV (COXIV) expression in red gastrocnemius. Actin is detected at ~ 42 kDa. Representative stains used for: **A)** cyt-*c* and ANT, **B)** COXIV.