

Examination of Voluntary Wheel Running and Skeletal Muscle Metabolism in the Sarcolipin Knock-Out Mouse

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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ABSTRACT

Sarcoplipin (SLN) is a small sarcoplasmic reticulum (SR) integral membrane protein that regulates the SR Ca^{2+} -ATPase (SERCA). Previous studies indicate that the functional interaction between SLN and SERCA is thermogenic in nature. Recently, SLN knock-out (SLNKO) mice have been shown to develop excessive obesity and glucose intolerance when placed on a high-fat diet (HFD; 42% kcal derived from fat) relative to wild-type (WT) littermates, implicating SLN in diet-induced obesity. The purpose of this thesis was two-fold: 1) to determine whether an excessively obese phenotype persists when SLNKO mice are given access to voluntary exercise, and 2) to determine if SLN ablation results in a deficit in skeletal muscle oxidative capacity, given the integral role cellular Ca^{2+} plays in mitochondrial metabolism. Mice were fed either standard chow or a HFD for 8 weeks, and remained sedentary or given access to voluntary running wheels during this period. Glucose tolerance was assessed pre- and post-diet, along with weight gain and adiposity. Skeletal muscle succinate dehydrogenase (SDH), citrate synthase (CS), cytochrome *c* oxidase (COX), and 3-hydroxyacyl CoA dehydrogenase (β -HAD) activities were measured in the soleus (SOL) and extensor digitorum longus (EDL) of both chow- and high-fat fed sedentary mice. Both average daily running distance and total exercise volume were not different between WT and SLNKO mice given voluntary running wheels. As before, sedentary SLNKO mice gained more mass following the HFD relative to WT counterparts ($P < 0.05$); however, no difference in mass gain existed between genotype for voluntary exercising mice on a HFD. Despite this, SLNKO animals were more obese and glucose intolerant following high-fat feeding, regardless of activity status ($P < 0.05$). Under chow-fed

conditions COX activity was higher in the EDL of SLNKO mice ($P < 0.05$), while no differences in SDH, CS, or β -HAD existed between genotype in either muscle group. Following the HFD, no changes in mitochondrial enzyme activities within the SOL existed. COX activity in the EDL remained elevated in SLNKO mice post-HFD ($P < 0.001$), while β -HAD increased in both WT and SLNKO animals relative to chow-fed controls ($P < 0.05$). These findings suggest that increasing energy expenditure through voluntary activity cannot compensate for increased basal SERCA Ca^{2+} -pumping efficiency during caloric excess. Additionally, ablation of SLN does not result in a metabolic deficit within skeletal muscle, nor does it limit the adaptive enzymatic response of SLNKO mice to high-fat feeding. Thus, the findings of this study provide further support of the view that SLN's thermogenic role is the primary mechanism of diet-induced obesity in SLNKO mice.

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DEDICATION

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Thesis Overview

The sarcoplasmic reticulum (SR) is the major storage site of Ca^{2+} within skeletal muscle. Upon sarcolemmal depolarization, Ca^{2+} is released from the SR and into the cytosol where it can interact with the contractile apparatus, allowing for muscle contraction to occur. In order for skeletal muscle to relax, the released Ca^{2+} must be re-sequestered within the SR, a process mediated by the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA) located within the SR membrane. In addition to initiating muscle relaxation, SERCA activity is responsible for maintaining a resting Ca^{2+} gradient $>10^4$ across the SR membrane (MacLennan, 1990). Maintenance of a low cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) by SERCA is an energy-dependent process; under optimal conditions the stoichiometry of Ca^{2+} pumping into the SR lumen is 2 mol of Ca^{2+} per mol of ATP hydrolyzed by SERCA, termed a 2:1 coupling ratio. This coupling ratio (i.e. the energetic efficiency of SERCA), along with SERCA activity, can be altered by the SERCA regulatory proteins phospholamban (PLN) and sarcolipin (SLN).

Experiments utilizing reconstituted lipid vesicles containing various molar ratios of SERCA and SLN have demonstrated that the interaction between these proteins is thermogenic in nature (Smith *et al.*, 2002; Mall *et al.*, 2006). Recently, ablation of SLN in mice has been shown to lead to increased susceptibility to diet-induced obesity and excessive whole-body glucose intolerance (Bombardier, 2010). Protection from this obese phenotype in wild-type (WT) mice is believed to result from a greater energetic requirement by SERCA to maintain basal cytosolic Ca^{2+} concentration due to SLN's uncoupling effect on Ca^{2+} uptake, as WT mice have a decreased coupling ratio relative to SLN knock-out (SLNKO) mice (i.e. require more energy to translocate a given amount of

Ca²⁺).

Several important questions still remain with respect to SLNKO mice and the development of diet-induced obesity and excessive whole-body glucose intolerance. Previous analyses of SLNKO mice have only occurred under sedentary conditions. Some have proposed that examination of the physiological relevance of a gene/protein towards a disease pathogenesis must occur under a condition of physiological stress such as exercise (Booth and Laye, 2009), as some transgenic mice only develop a disease phenotype when chronically sedentary (Huszar *et al.*, 1997; Haskell-Luevano *et al.*, 2009). Thus, it is important to examine whether the excessively obese phenotype of SLNKO mice following over-nutrition persists when given access to increased daily energy expenditure through ambulation (i.e. voluntary wheel running).

Additionally, although excessive obesity may result from the reduced cost of skeletal muscle Ca²⁺-handling in SLNKO mice, a reduction in mitochondrial content may also be at play. It is well established that Ca²⁺ is an important mediator of mitochondrial biogenesis within skeletal muscle (Chin, 2005), and that oxidative capacity is reduced in human obesity and type II diabetes mellitus (T2DM; Kelley *et al.*, 2002; Boushel *et al.*, 2007; Holloway *et al.*, 2007). Additionally, mitochondrial dysfunction has been implicated by some as a potential cause of skeletal muscle insulin-resistance associated with obesity (Kelley *et al.*, 2002). It is unclear what role, if any, SLN ablation has on Ca²⁺ signaling and mitochondrial biogenesis, and whether or not a reduction in skeletal muscle oxidative capacity is at least associated with the increased susceptibility to diet-induced obesity and reduced glucose tolerance of SLNKO mice. Thus, the main objectives of this study are two-fold:

- 1) examine the effect of voluntary wheel-running on obesity and glucose tolerance in SLNKO mice following high-fat feeding, and
- 2) examine whether ablation of SLN results in an altered skeletal muscle oxidative capacity under chow-fed conditions, and determine if an interactive effect exists between diet (chow vs. high-fat) and genotype on skeletal muscle oxidative capacity.

It is hypothesized that:

- 1) despite access to voluntary running wheels, a phenotype difference will still exist in SLNKO mice following high-fat feeding, and
- 2) ablation of SLN will not alter skeletal muscle oxidative capacity under both chow and high-fat conditions.

Introduction

Obesity and Type II Diabetes Mellitus in Canada

Obesity is a condition in which an excessive accumulation of visceral and subcutaneous fat results from a chronic energy imbalance, such that energy intake exceeds expenditure. According to the World Health Organization (WHO), individuals are obese if their body mass index (BMI: kg/m²) is ≥ 30 , while those with a BMI of 25-29 are considered overweight (WHO, 2000). Across Canada the prevalence of obesity has reached epidemic proportions; between 1985 and 1998 obesity prevalence has more than doubled from 5.6% to 14.8% (Katzmarzyk, 2002). A recent report indicates that nearly 35% of Canadians are overweight and that the trend of increasing obesity prevalence is continuing (Katzmarzyk and Mason, 2006). Alarming still is that this rate of increase is more rapid in adolescents than adults (Tremblay *et al.*, 2002).

The rise in obesity represents a significant financial burden to the Canadian healthcare system; it is estimated that treating obesity and related comorbidities accounts for approximately 2.4% (~\$1.8 billion) of total healthcare expenditures (Birmingham *et al.*, 1999). Many other diseases are associated with obesity such as various cancers, hypertension, cardiovascular disease, and type II diabetes mellitus (T2DM; WHO, 2000). Of these comorbidities, T2DM is of particular concern as approximately 90% of cases are linked to excessive body weight (Hossain *et al.*, 2007). Estimates indicate that by 2020 the total number of Canadians with diabetes will reach 3.7 million people, most of whom will be classified type II, as T2DM comprises approximately 90% of total diabetes cases (Canadian Diabetes Association, 2009). In Ontario alone, the prevalence of diabetes in 2005 has already surpassed the WHO estimated global prevalence rate for 2030

(Lipscombe and Hux, 2007).

T2DM is characterized by chronic hyperglycemia resulting from a diminished ability of insulin-sensitive tissues to respond to insulin stimulation (i.e. insulin resistance). The ability of individuals to maintain euglycemia, a narrow range of plasma glucose concentration between 80-100 mg/dl (Boden, 2001), requires coordination between several tissues, including the pancreas, liver, adipose tissue, and skeletal muscle. Individuals with T2DM first pass through a period of pre-diabetes, defined clinically as having impaired fasting glucose (100-125 mg /dl) or impaired glucose tolerance (140-200 mg/dl two hours post oral glucose challenge; Meigs *et al.*, 2003; Greg and Kriska, 2008). As T2DM progresses, insulin secretion decreases due to dysfunction of pancreatic β -cells (i.e. insulin producing cells; Gregg and Kriska, 2008), requiring patients to regulate glycemia through exogenous insulin administration. A hallmark feature of T2DM is skeletal muscle insulin resistance, which is believed to be a major contributor to the disease pathogenesis. Although the mechanisms leading to a reduction of skeletal muscle insulin sensitivity are complex, lipid oversupply resulting in the accumulation of intramuscular triglycerides (IMTG) is a key factor.

Lipid Induced Skeletal Muscle Insulin Resistance

Skeletal muscle comprises ~40% of body mass in adult humans (Rolfe and Brown, 1997), and is estimated to account for 70-80% of peripheral glucose uptake in response to an oral glucose challenge (DeFronzo *et al.*, 1981). Given the absolute proportion of skeletal muscle to body mass and its capacity for glucose disposal, reduced skeletal muscle insulin sensitivity will lead to dysregulation of glycemic control.

Elevated plasma free fatty acids (FFAs) are believed to be a major causal factor in the development of skeletal muscle insulin resistance leading to T2DM. Direct evidence for a role of elevated plasma FFAs have come from studies utilizing the euglycemic-hyperinsulinemic clamp technique with concomitant lipid infusion. These studies have shown a reduction in peripheral insulin sensitivity with physiological elevations in plasma FFAs in healthy individuals (Ferrannini *et al.*, 1983; Boden and Jadali, 1991), individuals predisposed to T2DM (Kashyap *et al.*, 2003), and those who are both obese and have T2DM (Boden and Chen, 1995; Roden *et al.* 1996). Development of insulin resistance with lipid oversupply is believed to result from the accretion of triglyceride and lipid species within skeletal muscle, which interfere directly with the insulin-signaling cascade.

Entrance of circulating FFAs into skeletal muscle is a protein-mediated process regulated by the integral membrane protein fatty acid translocase (FAT/CD36; Bonen *et al.*, 2004a). FAT/CD36 is sequestered within intracellular vesicles and migrates to the sarcolemma to increase lipid uptake in response to muscle contraction and insulin (Holloway *et al.*, 2008). Once FFAs cross the sarcolemma, they are subject to two major fates depending on cellular energy demand: 1) storage as IMTG or 2) mitochondrial oxidation. IMTG concentration is elevated in human obesity and T2DM (Bonen *et al.*, 2004b; He *et al.*, 2001), but IMTGs themselves are not believed to directly cause skeletal muscle insulin resistance. Similar to obesity and T2DM, highly trained athletes show elevated IMTG content but are insulin sensitive (Goodpaster *et al.*, 2001); this ‘athlete’s paradox’ is likely due to increased substrate turnover in trained individuals, preventing stagnation of IMTG and accumulation of lipid metabolites. However, intermediates of

lipid metabolism including diacylglycerols (Itani *et al.*, 2002), long-chain fatty acyl-CoAs (Cooney *et al.*, 2002), and ceremides (Chavez *et al.*, 2003) are implicated in altering post-receptor events of insulin signaling. In insulin resistant states, elevation of these lipid species inhibits intermediate and distal steps of the insulin-signaling cascade, resulting in reduced trafficking of glucose transporter-4 (GLUT-4) containing vesicles to the sarcolemma.

An ongoing point of contention is the mechanistic origin of IMTG accumulation seen in obesity and T2DM. A common observation in obese and diabetic muscle is a reduction in mitochondrial content (Kelley *et al.*, 2002; Boushel *et al.*, 2007; Holloway *et al.*, 2007). Microarray analysis of human diabetic skeletal muscle has also shown a coordinated down-regulation of genes involved in oxidative metabolism, including the peroxisome proliferator activator receptor γ co-activator 1 (PGC-1), the so-called “master regulator” of oxidative gene expression (Patti *et al.*, 2003). Some have proposed that reduced skeletal muscle oxidative capacity or mitochondrial dysfunction (i.e. an intrinsic defect in the mitochondria’s ability to catabolize carbon substrate) reduces fatty acid oxidation, resulting in IMTG accumulation (Kelley *et al.*, 2002; Patti *et al.*, 2003; Lowell and Shulman, 2005). However, it is yet unclear as to whether reduced mitochondrial content or mitochondrial dysfunction represents a cause or consequence of obesity and T2DM.

Conversely, others have found mitochondrial fatty acid oxidation not to be impaired in both human (Bonen *et al.*, 2004b; Boushel *et al.*, 2007; Holloway *et al.*, 2007) and rodent obesity (Holloway *et al.*, 2010). Additionally, skeletal muscle contain mitochondria in excess of what is needed to supply ATP to resting cells, and a reduction

in mitochondrial content to that seen in obesity and T2DM is unlikely to limit substrate metabolism (Hollozy, 2009). Instead, others propose that increased skeletal muscle lipid uptake results in the ectopic accumulation of IMTG. Although total expression of skeletal muscle FAT/CD36 protein is unchanged in human obesity and T2DM, sarcolemmal FAT/CD36 content is increased and associated with a 4-fold rise in skeletal muscle lipid uptake (Bonen *et al.*, 2004b). Similar findings have also been shown in Zucker obese rats and Zucker diabetic fatty rats (Luiken, *et al.*, 2001; Chabowski *et al.*, 2006). Thus, mounting evidence suggests that insulin resistant muscle has an increased capacity to extract circulating FFAs due to a redistribution of FAT/CD36 from the intracellular compartment to the plasma membrane. Methods to improve skeletal muscle insulin sensitivity could involve blockade of lipid entry into muscle, or increasing IMTG turnover by augmenting cellular energy demands. Increasing energy demand can be achieved through exercise or reducing the efficiency of several ATP consuming processes that contribute to basal metabolism. The latter scenario involves several adaptive mechanisms possessed by organisms to “waste” excess energy, including shivering and non-shivering thermogenesis.

Mechanisms of Shivering and Non-Shivering Thermogenesis

Adaptive thermogenesis refers to an increase in heat production (i.e. energy utilization) by an organism in response to changes in environmental temperature or diet (Lowell and Spiegelman, 2000). The ability of an organism to alter energy expenditure in response to periods of food shortage/excess and temperature is an adaptive mechanism needed to maintain appropriate cellular energy stores and body temperature. Metabolic

heat is derived from various cellular reactions including protein synthesis, ATP synthesis, and those needed to actively maintain cellular ions gradients. Dysfunction in energy expending processes, particularly during periods of increased caloric intake, is likely to contribute to obesity. Several known mechanisms exist to increase heat production, including skeletal muscle contraction (e.g. shivering thermogenesis) and mitochondrial proton leak (e.g. non-shivering thermogenesis).

Skeletal muscle contraction/relaxation cycling is an energy-dependent process involving two major sites of energy consumption, namely by myosin ATPase and SERCA. The electrical and mechanical processes involved in skeletal muscle excitation-contraction (EC) coupling are complex and involve a number of protein interactions. EC coupling refers to the processes linking sarcolemmal depolarization to the release of intracellular Ca^{2+} stores, resulting in actin-myosin crossbridge formation and force development (Dulhunty, 2006). Upon stimulation by a motor neuron, an action potential travels along the sarcolemmal surface and down the transverse-tubule (t-tubule), where a voltage change in this region is sensed by dihydropyridine receptors (DHPRs) located in the t-tubular membrane. In skeletal muscle, DHPRs physically interface with ryanodine receptors (RyR) located on the terminal cisternae of the SR, together forming calcium release units (Franzini-Armstrong and Protasi, 1997). A voltage induced conformational change in the DHPRs result in the opening of RyRs, causing efflux of stored Ca^{2+} from the SR into the cytosol. Rising $[\text{Ca}^{2+}]_f$ enables interaction between actin and myosin filaments by Ca^{2+} binding to troponin C on the thin filament and removing the steric inhibition of tropomyosin. Upon hydrolysis of ATP by myosin ATPase, crossbridge formation occurs between actin and myosin filaments allowing for muscle contraction

and heat generation. In order for skeletal muscle to then relax, $[Ca^{2+}]_i$ must be sequestered back within the SR, a process mediated by SERCA. Although activation of skeletal muscle through exercise is an obvious way to utilize excess energy, several non-shivering thermogenic mechanisms may be more important during resting and post-prandial conditions in regulating energy balance.

A well-characterized mechanism of adaptive non-shivering thermogenesis is that of mitochondrial proton leak in brown adipose tissue (BAT) mediated by uncoupling protein-1 (UCP-1). UCP-1 is an inner mitochondrial membrane protein that causes protons to leak into the mitochondrial matrix from the intermembrane compartment, with energy being dissipated as heat (Klingenberg and Huang, 1999; Lowell and Spiegelman, 2000). Disruption of the mitochondrial H^+ gradient by UCP-1 uncouples protonmotive force from ATP synthesis; thus, more carbon substrate must be oxidized to produce reducing equivalents (i.e. NADH and $FADH_2$) and re-establish the H^+ gradient, a process further releasing heat. Initial examination of the UCP-1 knockout mouse indicated sensitivity to cold-exposure, but unexpectedly these mice did not become obese under chow or high-fat conditions, which was attributed to a compensatory increase in UCP-2 within BAT (Enerback *et al.*, 1997). However, a recent reexamination of this model under thermally neutral conditions ($\sim 30^\circ C$; i.e. temperatures at which no compensatory mechanisms are needed to regulate body temperature) indicate that lack of UCP-1 results in pronounced obesity under both chow and high-fat conditions (Feldmann *et al.*, 2009). The study of BAT mitochondrial uncoupling in rodents at thermoneutrality has reestablished UCP-1 as a candidate gene involved in the pathogenesis of obesity.

Until recently, humans were believed to contain no BAT past adolescence. Using

fluorodeoxyglucose (FDG) positron emission tomography, humans exposed to cold show enhanced FDG uptake in small areas of the supraclavicular and neck regions, which is inhibited by β -adrenergic blockade, a known activator of UCP-1 induced thermogenesis (Nedergaard *et al.*, 2007). Subsequently, direct evidence for the presence of BAT in humans has come from positive staining of UCP-1 immunoreactive adipose tissue harvested from patients undergoing surgery for thyroid diseases (Zingaretti *et al.*, 2009). Despite the now confirmed presence of BAT in man, its physiological significance in thermogenesis and obesity-prevention has yet to be determined. Although BAT is present in small amounts, skeletal muscle comprises a large proportion of overall body mass, making it an important thermogenic target tissue in man.

Skeletal muscle of humans and rodents contain UCP-3, which shares considerable sequence homology with UCP-1 and 2 (Vidal-Puig *et al.*, 1997). Similar to initial studies of UCP-1 knockout mice, ablation of UCP-3 in mice did not cause obesity under chow or high-fat conditions (Gong *et al.*, 2000; Vidal-Puig *et al.*, 2000); lack of a genotype difference in these studies is likely due to the occurrence of thermoregulatory compensation in the transgenic animals given that housing conditions were below thermoneutrality. However, skeletal muscle-specific overexpression of UCP-3 in mice does attenuate the development of an obese phenotype following high-fat feeding (Son *et al.*, 2004), demonstrating the importance of skeletal muscle thermogenesis in protecting against excessive obesity. Several other processes within skeletal muscle may contribute to adaptive thermogenesis, including futile cycling of fatty acid synthesis and oxidation (Solinas *et al.*, 2004), protein and metabolite phosphorylation/dephosphorylation cycles, and ion leakage, particularly Ca^{2+} leakage from the SR (Lowell and Spiegelman, 2000).

Ca²⁺ Cycling and Adaptive Thermogenesis

The contribution of skeletal muscle Ca²⁺ pumping to thermogenesis is evident in the genetic disorder malignant hyperthermia, in which a hypermetabolic state results from an RyR mutation causing excessive Ca²⁺ release during inhalational anesthesia (Denborough, 1998; MacLennan *et al.*, 1990). Continual Ca²⁺ release results in the activation of myosin ATPase and SERCA, causing excessive contracture and heat generation. Furthermore, evidence for futile cycling of Ca²⁺ as a thermogenic mechanism is seen in ‘heater’ organs of deep-sea diving fish, which are comprised of specialized muscle cells containing a dense t-tubular and SR network without myofilaments in order to regulate brain and eye temperature during diving (Morrissette *et al.*, 2003). Given that BAT is minimal in larger mammals and that the role of UCP-3 within skeletal muscle is incompletely understood, altering the occurrence of SR Ca²⁺ leakage or the energetic cost to pump Ca²⁺ may be an important mechanism of adaptive thermogenesis, and a primary candidate for this role is SERCA.

SERCA pumps are SR integral membrane proteins that catalyze the ATP-dependent transport of Ca²⁺ from the cytosol into the SR lumen (MacLennan, 1990). The major functions of SERCAs are to initiate muscle relaxation and maintain SR Ca²⁺ load in resting cells despite a Ca²⁺ gradient >10⁴ across the SR membrane (MacLennan, 1990; Toyoshima, 2008). Under optimal conditions, 2 Ca²⁺ ions are pumped into the SR lumen per molecule of ATP hydrolyzed (i.e. 2:1 coupling ratio), as SERCA contains two Ca²⁺ binding sites and one ATP binding site based upon its crystal structure (Toyoshima *et al.*, 2000). Several SERCA isoforms exist in mammalian muscle, with SERCA1a being

expressed predominantly in fast-twitch skeletal muscle, while SERCA2a is the major isoform found in slow-twitch skeletal muscle and the heart (Wu and Lytton, 1993; Tupling, 2009).

Given that Ca^{2+} transport is energy-dependent and that $[\text{Ca}^{2+}]_f$ must be maintained at 100 nM despite a gradient favoring SR Ca^{2+} efflux, SERCA activity likely plays an integral role in resting skeletal muscle energy expenditure. Several studies have estimated or directly measured the energetic contribution of SERCA and have yielded results ranging from 3.4-24% of basal metabolism (Clausen *et al.*, 1991; Hasselbach and Oetliker, 1983; Chinet *et al.*, 1992; Dulloo *et al.*, 1994). These results suggest that a significant amount of SR Ca^{2+} leakage occurs under resting conditions that must continually be rectified by SERCA activity. Since SERCA accounts for a potentially large proportion of resting energy expenditure, lowering its contribution may be implicated in the development of diet-induced obesity. Two SERCA regulatory proteins have been shown to alter the energetic cost of SR Ca^{2+} transport, namely PLN and SLN.

Regulation of SERCA by Phospholamban (PLN) and Sarcolipin (SLN)

PLN and SLN are small SR integral membrane proteins that physically interact with SERCA to regulate Ca^{2+} uptake activity (MacLennan *et al.*, 2003). PLN is a 52 amino acid proteolipid containing a larger cytoplasmic N-terminal domain anchored to the SR by a C-terminal transmembrane domain (Fujii *et al.*, 1987). SLN is a smaller 31 amino acid proteolipid consisting of a 7 amino acid N-terminal domain, a 19 amino acid transmembrane domain, and a 5 amino acid C-terminal domain that protrudes into the SR lumen (Odermatt *et al.*, 1997). Expression of PLN and SLN are both tissue and species

specific (Babu *et al.*, 2007; Odermatt *et al.*, 1997; Vangheluwe *et al.*, 2005). In mice PLN protein expression is high in the ventricle, whereas SLN protein expression is greater in the atria and soleus (SOL), and to a lesser extent in the extensor digitorum longus (EDL; Vangheluwe *et al.*, 2005; Bhupathy *et al.*, 2007; Bombardier, 2010). Currently, little is known about the protein expression patterns of SLN in human tissues due to the absence of an antibody against human SLN. However, Northern blot analysis indicates that SLN mRNA is abundant in human skeletal muscle (Odermatt *et al.*, 1997).

Based upon similarities in gene sequence and conserved regions in the N-terminal and transmembrane domains, PLN and SLN are considered to be homologous proteins (Odermatt *et al.*, 1997). When bound to SERCA, PLN and SLN reduce its apparent affinity for Ca^{2+} in cardiac (Kadambi *et al.*, 1996; Babu *et al.*, 2006; Gramolini *et al.*, 2006) and skeletal muscle (Tupling *et al.*, 2002; Bombardier, 2010). Additionally, a distinct functional feature of SLN is its ability to reduce SERCA maximal Ca^{2+} uptake activity despite increasing $[\text{Ca}^{2+}]_f$ (Tupling *et al.*, 2002; Bhupathy *et al.*, 2007).

PLN and SLN action are governed by reversible phosphorylation and dephosphorylation control by protein kinases/phosphatases. Their inhibitory effect on SERCA is removed by protein kinase activation, resulting in their phosphorylation and physical dissociation from SERCA. Cyclic-AMP dependent protein kinase (PKA) and Ca^{2+} -calmodulin-dependent protein kinase II (CaMKII) are known to phosphorylate PLN at serine-16 and threonine-17, respectively, during β -adrenergic stimulation (Bhupathy *et al.*, 2007). The conserved threonine-5 residue of SLN is also phosphorylated by CaMKII (Bhupathy *et al.*, 2009). Thus, during activation of Ca^{2+} dependent signaling pathways (e.g. muscle contraction, β -adrenergic stimulation), Ca^{2+} handling and force

characteristics are improved in part by removal of the inhibitory action of PLN and SLN on SERCA activity. Despite the well-documented role of PLN and SLN in mediating the β -adrenergic response of the heart and cardiac contractility, less is known about their physiological significance in skeletal muscle, particularly under resting conditions in which a physical association with SERCA exists. Recently, SLN has been implicated in skeletal muscle adaptive thermogenesis.

SLN Ablation in Adaptive Thermogenesis and Diet-Induced Obesity

The interaction between SERCA and SLN has been shown to be thermogenic in nature. Experiments using reconstituted vesicles containing both SLN and SERCA demonstrate that SLN uncouples Ca^{2+} transport from ATP hydrolysis and increases the proportion of energy released as heat per mol of ATP hydrolyzed (Smith *et al.*, 2002; Mall *et al.*, 2006). In agreement with this, the apparent coupling ratio (i.e. Ca^{2+} transported: ATP hydrolyzed) in the SOL of SLNKO mice is higher relative to that of WT littermates (Bombardier, 2010). The higher coupling ratio in SLNKO mice is the result of lower Ca^{2+} ATPase activity, indicating that in the absence of SLN, SERCA consumes less energy to establish a given Ca^{2+} gradient across the SR, making it energetically more efficient. At the whole-body level, increasing the energetic efficiency of SERCA results in a predisposition to diet-induced obesity, as SLNKO mice become more obese than WT littermates following an eight-week high-fat diet (Bombardier, 2010). SLN protein expression within SOL of WT mice also increases following high-fat feeding (Bombardier, 2010), suggesting that SLN is regulated partly by diet. Increased SLN expression would be expected to lead to a concomitant decrease in the energetic

efficiency of SERCA, as this may be the major factor preventing excessive weight gain in WT mice following overnutrition. Accompanying the development of excessive obesity in SLNKO mice is a reduction in whole-body glucose tolerance (Bombardier, 2010); however, whether this represents a reduction in skeletal muscle insulin sensitivity as yet remains unclear. Together, these data indicate that altering basal Ca^{2+} handling energetics by reducing the energetic efficiency of SERCA through its interaction with SLN is a mechanism of adaptive thermogenesis. Therefore, reduced skeletal muscle SLN protein expression may be a pre-disposing factor to the development of diet-induced obesity and T2DM. However, it should be noted that excessive obesity in the SLNKO model has only been demonstrated when mice remain sedentary, which negates the increase in energy expenditure associated with daily ambulation. It is not yet clear whether excessive obesity will persist if these mice are given access to voluntary exercise.

Effects of Physical Inactivity on Oxidative Metabolism and Insulin Sensitivity

The human genome was selected for its capacity to complete physical activity and store energy, as this would confer a selective advantage during foraging, physical confrontation, and periods of food shortage (Booth and Lees, 2007). However, with the advent of modern technologies, a need to engage in a high volume of physical activity is not obligatory for survival. Thus, the combination of a genome specialized for energy storage with an environment that accommodates physical inactivity and has *ad libitum* access to food, fosters the development of chronic diseases like obesity and T2DM (Booth *et al.*, 2008). Despite sedentary behaviour being a cause of chronic illness, little is

known about how lack of physical activity contributes to diseased states (Booth *et al.*, 2008). Skeletal muscle oxidative capacity in trained cyclists and runners declines rapidly in the initial 20 days following 10-24 months of intense training (Chi *et al.*, 1983). Additionally, cessation of physical activity elicits a rapid decline of insulin action in highly trained athletes, with whole-body insulin sensitivity decreasing to that of sedentary individuals ~1.5-2.5 days following their last exercise bout (Burstein *et al.*, 1985; Oshida *et al.*, 1991). Despite these studies indicating that maintenance of a high mitochondrial content and insulin sensitivity necessitates regular physical training, little is known about how decreased daily physical activity, not exercise training *per se*, contributes to augmented oxidative capacity and insulin resistance of skeletal muscle.

Booth and colleagues (Kump and Booth, 2005a/b) have utilized a voluntary running wheel model to mimic ambulatory activity of rodents and examine how reduced daily physical activity alters skeletal muscle insulin sensitivity and skeletal muscle energy metabolism. Upon removing access of male Fischer-Brown Norway rats to voluntary activity by locking running wheels, skeletal muscle submaximal insulin-stimulated 2-deoxyglucose uptake was reduced to that of sedentary animals after 53 hours of physical inactivity (Kump and Booth, 2005a). This was accompanied by reduced insulin binding to the insulin receptor β subunit, alterations in the phosphorylation status of insulin signaling intermediates, and reduced GLUT-4 protein expression. Additionally, the rate of triacylglycerol synthesis into epididymal fat was increased above sedentary animals just 10 hours following cessation of activity, and preceded increases in visceral adiposity with further inactivity (Kump and Booth, 2005b). Skeletal muscle fatty acid oxidation decreased to that of sedentary animals after 173 hours following wheel lock, and was

accompanied by a similar decrease in PGC-1 α mRNA expression (Laye *et al.*, 2009). Together, these studies indicate that removal of daily ambulation for a short period is enough to acutely reduce skeletal muscle mitochondrial function and insulin sensitivity to that of habitually sedentary individuals. Although the signaling mechanism(s) mediating the physiological changes with reduced physical activity are unclear, it is evident that regular daily activity is necessary for the maintenance of metabolic function and the overall metabolic health of an organism.

It is clear from the above studies that removing access to ambulation in rodent models can have drastic and immediate effects on normal physiological function. Some have argued that in order to determine the contribution of a gene/protein to a disease pathogenesis, examination of that gene/protein should occur under a condition of physiological stress (Booth and Laye, 2009). To this end, transgenic models may display divergent phenotypes under conditions of increased physical activity relative to sedentary counterparts. For example, melanocortin-4 receptor knockout mice are obese, hyperglycemic, hyperinsulinemic and hyperphagic when sedentary (Huszar *et al.*, 1997), but when given access to voluntary exercise this obese phenotype is abolished (Haskell-Luevano *et al.*, 2009). Therefore, in specific instances increasing energy expenditure through voluntary exercise can compensate for a genetic predisposition to obesity. Thus, it is of extreme interest to examine whether an excessively obese phenotype persists in SLNKO mice fed a high-fat diet and given access to voluntary exercise. While physical inactivity can pre-dispose individuals to obesity and diabetes, so too could a reduction in skeletal muscle oxidative capacity or mitochondrial function (discussed above). Although protection from diet-induced obesity may be due to SLN's thermogenic effect,

it is not known what effect, if any, SLN ablation has on skeletal muscle oxidative metabolism. This is important to examine given the relationship between Ca^{2+} signaling and mitochondrial biogenesis.

The Role of Skeletal Muscle Ca^{2+} in Oxidative Metabolism

Increases in skeletal muscle mitochondrial content (i.e. oxidative capacity) is a well-documented adaptation to exercise training, but the exact mechanism(s) and signal(s) leading to such an increase have remained elusive until relatively recent. It is now understood that cellular Ca^{2+} plays an integral role in regulating skeletal muscle mitochondrial biogenesis. Initial experiments of L6 myotubes showed increased mRNA expression of nuclear encoded cytochrome *c* after continuous treatment with the Ca^{2+} ionophore A23187 for 48 hours (Freysenet *et al.*, 1999). Similarly, intermittent treatment of L6 myotubes with other Ca^{2+} mobilizing agents such as caffeine (an RyR agonist) resulted in increased protein expression of various mitochondrial markers including citrate synthase, cytochrome *c*, aminolevulinate synthase, cytochrome *c* oxidase subunit 1, and was accompanied by increased mitochondrial function (Ojuka *et al.*, 2002; Ojuka *et al.*, 2003). Caffeine exposure also increased protein expression of PGC-1, nuclear respiratory factors-1/2, and mitochondrial transcription factor A (Ojuka *et al.*, 2003), of which are needed to coordinate the transcriptional activation of the nuclear and mitochondrial genomes. These exercise-mimicking effects are inhibited by the addition of Ca^{2+} chelating agents such as EGTA and the RyR blocker dantrolene (Ojuka *et al.*, 2002; Ojuka *et al.*, 2003). However, the $[\text{Ca}^{2+}]_f$ reached in these studies may be supraphysiological, and flooding the cytosol with Ca^{2+} for prolonged periods does not mimic the characteristic Ca^{2+} oscillations during electrical stimulation and exercise.

In vivo evidence for Ca²⁺-dependent signaling and mitochondrial biogenesis has come from several transgenic mouse models. Mice expressing a constitutively active form of CaMKIV in skeletal muscle show a transition towards a more oxidative phenotype (Wu *et al.*, 2002), as do mice overexpressing an activated form of the Ca²⁺/calmodulin-dependent phosphatase calcineurin (Chin, 2004). Additionally, ablation of the rodent Ca²⁺ buffering protein parvalbumin (PV), found predominantly in fast-twitch muscles, results in increased mitochondrial content and greater fatigue resistance of fast-twitch muscles (Chen *et al.*, 2001; Racay *et al.*, 2006). Conversely, mice overexpressing PV in slow-twitch muscles show lower succinate dehydrogenase activity (Chin *et al.*, 2003). Together, these studies indicate a Ca²⁺-dependence of mitochondrial biogenesis. Interestingly, alteration of resting (i.e. non-exercising) Ca²⁺-handling dynamics in PV transgenic mice is enough to modify mitochondrial content. Thus, it is of interest to determine whether SLN ablation augments skeletal muscle oxidative capacity.

Study Rationale

Although SLN has been implicated in the development of diet-induced obesity, previous studies of SLNKO mice have only occurred under sedentary conditions. In order to determine the functional impact SLN ablation has on susceptibility to obesity, further examination of SLNKO mice should occur under conditions of regular access to physical activity. Additionally, it is yet unknown whether altering basal Ca²⁺ handling through SLN ablation results in an inherent defect in skeletal muscle oxidative metabolism.

Study Objectives and Hypotheses

Objectives:

- 1) examine the effect of voluntary wheel-running on obesity and glucose tolerance in SLNKO mice following high-fat feeding, and
- 2) examine whether ablation of SLN results in an altered skeletal muscle oxidative capacity under chow-fed conditions, and determine if an interactive effect exists between diet (chow vs. high-fat) and genotype on skeletal muscle oxidative capacity.

Hypotheses:

- 1) given that SLN reduces the energetic efficiency of SERCA under basal conditions, a phenotype difference will still exist in SLNKO mice having access to voluntary running wheels following high-fat feeding, as SLN could also reduce SERCA efficiency during physical activity, and
- 2) ablation of SLN will not alter skeletal muscle oxidative capacity under both chow and high-fat conditions.

Methods

Experimental Animals and Genotyping

Transgenic mice lacking sarcolipin (SLN-null) were generously donated by Drs. David MacLennan and Anthony Gramolini (University of Toronto) and used to establish a breeding colony at the University of Waterloo. SNL-null mice were crossbred with C57BL/6J mice to generate heterozygous SLN-null breeding pairs. Breeding pairs yielded homozygous sarcolipin knockout (SLNKO, -/-), heterozygous (HET, +/-) and homozygous wild type (WT, +/+) mice. At four weeks of age ear clippings were taken, digested, and DNA was extracted for identification of mouse genotype using a commercially available kit (PureLink DNA Mini Kit; Invitrogen). Extracted DNA was subjected to PCR for amplification of target DNA. Briefly, ~50 ng of extracted DNA was added to a Taq DNA polymerase mixture (Fermentas) containing 3 mM MgCl₂, 200 μM dNTP, 10x Taq buffer (containing 750 mM Tris-HCL, 200 mM (NH₄)₂SO₄, and 0.1% (v/v) Tween 20), 1.5 μL Taq DNA polymerase, and 0.4 μM of appropriate forward and reverse primers (SLN-WT forward: 5'-TGT CCT CAT CAC CGT TCT CCT-3', SLN-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'). DNA was placed in a thermal cycler (S1000 Thermal Cycler; Bio-Rad) and denatured at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, extension at 72°C for 60 sec, and a final extension at 72°C for 7 min. Amplified target DNA was then resolved by 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).

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

Whole-Body Metabolic Rate and Experimental Diets

Prior to the initiation of pre-diet whole-body metabolic measurements, mice were acclimated to clear plastic cages with wire mesh bottoms for one week and given *ad libitum* access to water and powdered rodent chow (22/5 Rodent Diet (w) 8640; Harlan Teklad, Madison, WI). Whole-body metabolic measurements were made using a Comprehensive Laboratory Animal Monitoring System (CLAMS; Oxymax Series; Columbus Instruments, Columbus, OH). The CLAMS is an open-circuit indirect calorimeter capable of measuring O₂ consumption rate ($\dot{V}O_2$), food consumption, and cage activity on twelve experimental animals. Mice were housed individually in clear plexiglass cages (20 cm x 10 cm x 12.5 cm) in a temperature-controlled room (~22°C) under a reverse light/dark cycle (12/12 hr) with *ad libitum* access to water and powdered food (as above). Mice were acclimated to the CLAMS 24 hr before commencing measurements, after which data collection proceeded for 24 hr. CLAMS measurements were repeated three times on three separate weeks before and after administration of the experimental diet (**Figure 1**). Mice were not acclimated to clear plastic cages before commencing post-diet measurements, and those allocated to the high-fat diet (HFD) were given powdered high-fat food while in the CLAMS instead of standard rodent chow.

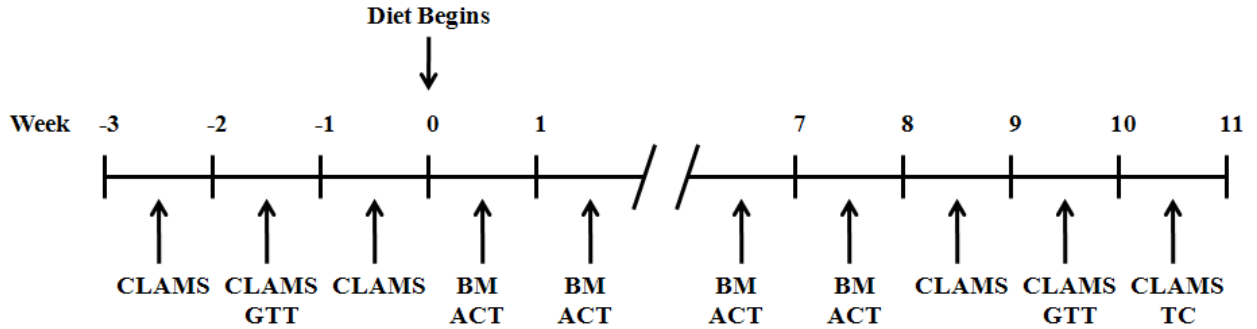


Figure 1. Experimental timeline (CLAMS: comprehensive laboratory animal monitoring system; GTT: glucose tolerance test; BM: body mass; ACT: activity; TC: tissue collection).

SLNKO and WT mice were randomly assigned to dietary and activity treatments as outlined below (**Figure 2**). The 8-week experimental diet consisted of *ad libitum* access to water and either a HFD containing 42% of kcal from fat (product TD 88137; Harlan Teklad, Madison, WI), or standard rodent chow, during which body mass was monitored weekly. Mice allocated to the exercise groups had voluntary running wheels placed in their cages, while those in the sedentary groups had their running wheels locked throughout the diet. Voluntary running was monitored daily with a magnetic sensor placed above the running wheels and wheel revolutions were recorded on an electronic counter. Total exercise volume (measured as the total distance travelled over the 8 week diet) was determined by multiplying total wheel revolutions by the running wheel circumference (40 cm).

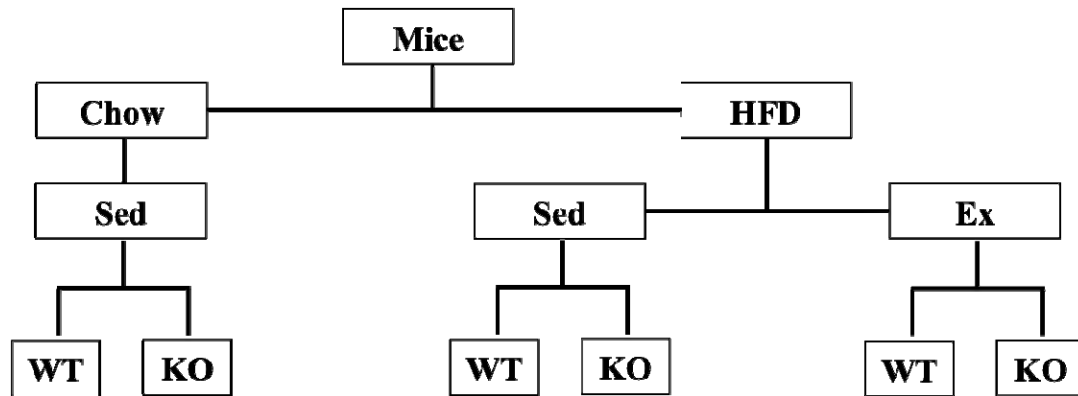


Figure 2. Experimental treatment design; HFD: high-fat diet; Sed: sedentary; Ex: exercise; WT: wild type; KO: sarcolipin knock-out.

Whole-Body Glucose Tolerance Tests and Tissue Collection

Whole-body glucose tolerance was assessed pre- and post-diet according to Li *et al.* (2000). Briefly, mice were fasted overnight (16 hr); running wheels of mice allocated to exercise treatments were also locked during the fasting period. Following an intraperitoneal injection of 20% D-glucose (1g/kg body mass), venous blood (5–10 μ L) was drawn from a tail vein and blood glucose was measured using a glucometer (Accu-Chek Aviva; Roche Diagnostics) immediately before, and at 30, 60 and 120 min post-injection.

Four hours prior to tissue collection, mice were fasted and access to voluntary exercise was removed. Experimental animals were euthanized by an anesthetic overdose of pentobarbital sodium (0.65 mg/kg body weight) and the SOL and EDL muscles were immediately excised, dissected free of connective tissue and visible fat and weighed. Skeletal muscles were then frozen in liquid N₂ and stored at -80°C until enzymatic analysis (described below). Liver, epididymal/inguinal and retroperitoneal fat pads were also removed, cleaned from surrounding tissues and weighed. An adiposity index was

calculated from the combined masses of the epididymal/inguinal and retroperitoneal fat pads according to Taylor and Phillips (1996) as:

$$(\text{sum of combined fat pad masses/body mass}) \times 100.$$

Biochemical Analysis

Muscle samples were homogenized 1:50 (w/v) in ice-cold phosphate-glycerol buffer (containing 16 mM Na₂HPO₄, 4 mM KH₂PO₄, 0.02% bovine serum albumin (BSA), 5 mM 2-mercaptoethanol, and 0.5 mM EDTA) using a glass homogenizer and stored at -80°C. Representative enzymes of the major biochemical pathways involved in energy metabolism were chosen for analysis. These included citrate synthase (CS) and succinate dehydrogenase (SDH) for the citric acid cycle, cytochrome c oxidase (COX) for the electron transport chain, 3-hydroxyacyl-CoA dehydrogenase (β-HAD) for β-oxidation, phosphofructokinase (PFK) and lactate dehydrogenase (LDH) for glycolysis, and hexokinase (HEX) for glucose phosphorylation. Maximal activities of all enzymes except COX were measured using NAD⁺/NADH-linked fluorometric end-point assays at room temperature (~22°C). All samples were measured in triplicate from the same phosphate-glycerol homogenate. SDH and PFK were measured on freshly homogenized tissue to avoid loss of enzyme activity with repeated freeze/thawing; all other enzymes were examined on thawed homogenate, as they are not affected by repeated thawing and storage at -80°C (Chi *et al.*, 1983; Henriksson *et al.*, 1986). Assay procedures for all enzymes except COX are published elsewhere (Chi *et al.*, 1983; Henriksson *et al.*, 1986). Total protein concentration of tissue homogenates was measured using the BCA procedure (Sigma).

COX activity was measured using a reaction mixture consisting of 970 μl of 10

mM potassium phosphate buffer and 20 μ l of reduced cytochrome C (Sigma C-2506) at 37°C. The original muscle homogenate was diluted 10:1 using 10 mM potassium phosphate buffer, creating a total dilution of 1:500 of the original homogenate. The reaction was initiated by adding 10 μ l of dilute homogenate to the reaction mixture, and the decrease in absorbance at 550 nm was measured spectrophotometrically for 3 min. COX activity was calculated by using the measured slope and the millimolar extinction coefficient of reduced cytochrome C at 550 nm, and the units of activity per gram of tissue was calculated and converted to units per gram protein.

Statistical Analysis

Weekly running, change in mass, glucose tolerance tests, and CLAMS variables were measured by 3- and 2-way repeated measures analysis of variance (ANOVA) where appropriate. Morphometric and enzymatic data were examined using a 2-way ANOVA. When appropriate, post-hoc comparisons were made using a Newman-Keuls test to examine specific mean difference. A 2-tailed Student's t-test was used to analyze basal (i.e. chow-fed) enzyme data and total exercise volume. All correlations with total exercise volume were done using a Pearson's correlation. Lastly, planned comparisons were made using a 1-tailed Student's t-test on variables previously examined by Bombardier (2010). Data are presented as mean \pm standard error (S.E.). Statistical significance was considered at $\alpha = 0.05$. Results were considered a trend at $0.10 \geq P > 0.05$.

Results

Voluntary Running Activity and Dietary Mass Gain

Voluntary running activity of high-fat fed animals is illustrated in **Figure 3**. Both average daily distance (**Figure 3A**) and total distance travelled over 8 weeks (**Figure 3B**) were highly variable. No differences between WT and SLNKO mice in average daily running existed during the 8 weeks, although there was a main effect of time ($P < 0.0001$), with running activity during weeks 2 to 7 greater than week 1 (**Figure 3A**). When activity was expressed as total distance travelled (**Figure 3B**), no genotype difference existed.

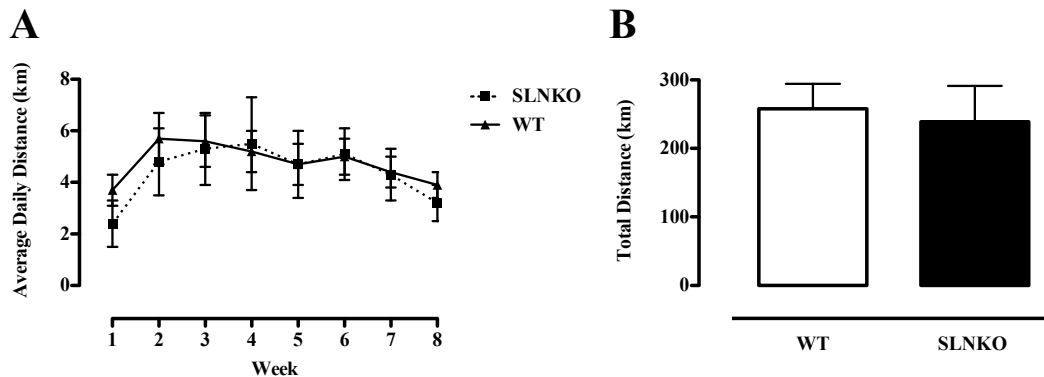


Figure 3. Voluntary running activity of high-fat fed wild-type (WT, $n = 14$) and sarcolipin knock-out mice (SLNKO, $n = 8$). A) Average daily distance run (km) over 8 weeks. A main effect of time existed ($P < 0.0001$). B) Total distance run (km) during the 8 week diet. Values are mean \pm S.E.

Pre-diet body mass was similar between WT ($n = 46$) and SLNKO ($n = 30$) mice (32.3 ± 0.44 g vs. 32.0 ± 0.57 g, respectively). Mass gain of sedentary chow-fed mice increased slightly over the 8 weeks (**Figure 4A**; main effect of time: $P < 0.0001$), but was not different between genotype. High-fat feeding resulted in significant ($P < 0.0001$)

mass gain over the 8 weeks in both WT and SLNKO mice, with SLNKO mice gaining more mass than WT counterparts between weeks 2 and 8 (**Figure 4A**; $P < 0.001$).

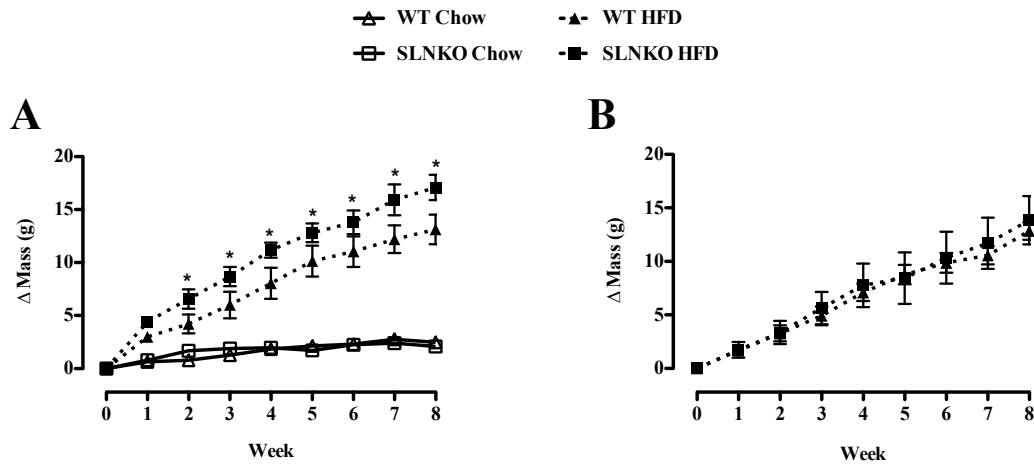


Figure 4. Change in initial body mass (g) of wild-type (WT) and sarcolipin knock-out (SLNKO) mice during 8 weeks of a chow or a high-fat diet (HFD). A) Sedentary mice (WT Chow: $n = 11$, SLNKO Chow: $n = 7$, WT HFD: $n = 9$, SLNKO HFD: $n = 7$). B) Mice given access to voluntary running wheels (WT HFD: $n = 14$, SLNKO HFD: $n = 8$). A significant main effect of time existed ($P < 0.0001$). Values are mean \pm S.E. * Significantly different than corresponding WT ($P < 0.001$).

Similar to sedentary mice, mass gain of high-fat fed mice given access to voluntary running wheels increased ($P < 0.0001$) throughout the diet, but no genotype difference existed (**Figure 4B**).

To determine whether voluntary running could attenuate mass gain as a result of the HFD, high-fat fed sedentary and exercising mice were compared using a 3-way repeated-measures ANOVA (**Figure 5**). No difference in mass gain between genotype was seen, although it increased over the course of the diet (main effect of time; $P < 0.001$). However, mice given access to voluntary running wheels gained less mass than sedentary counterparts (main effect of activity; $P < 0.05$). Separate planned comparisons were done to determine the effect of running wheel access on mass gain within each genotype. For WT mice, no differences were found over the 8 weeks; however, SLNKO

mice given running wheels tended to gain less mass (trend towards main effect of exercise: $P = 0.10$).

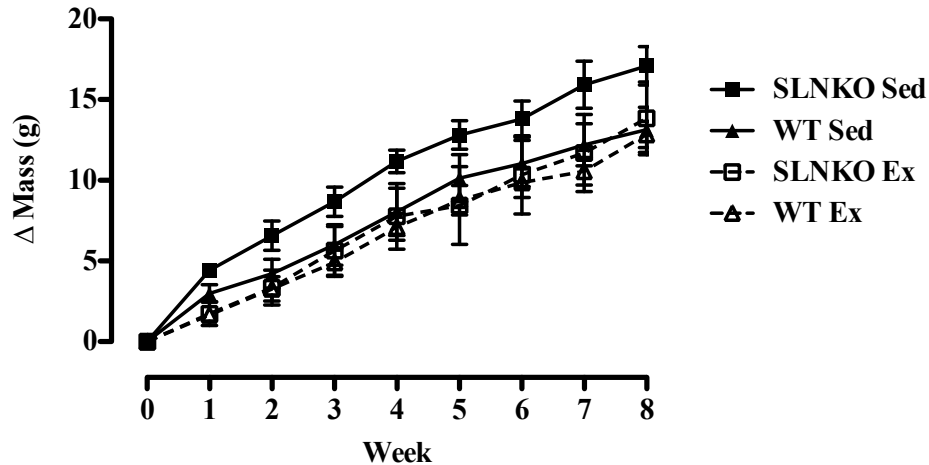


Figure 5. Change in initial body mass (g) during 8 weeks of high-fat feeding of wild-type (WT) and sarcolipin knock-out (SLNKO) mice. Animals were sedentary (Sed) or given access to voluntary running (Ex). A significant main effect of week ($P < 0.0001$) and activity ($P < 0.05$) existed, with Sed $>$ Ex. (WT Sed: $n = 9$, SLNKO Sed: $n = 7$, WT Ex: $n = 14$, SLNKO Ex: $n = 8$). Values are mean \pm S.E.

Adiposity and Anthropometric Measurements

In sedentary mice, high-fat feeding significantly increased adiposity index relative to chow-fed controls (**Figure 6A**; main effect of HFD: $P < 0.0001$), but no genotype difference existed under either dietary condition. Similarly, epididymal/inguinal fat pad mass was larger following the HFD (**Figure 6B**; main effect of diet: $P < 0.05$), with no genotype differences under either diet condition; however, planned comparisons of high-fat fed animals (Student's 1-tailed t-test) revealed a trend ($P = 0.06$) towards greater epididymal/inguinal fat pad mass in SLNKO mice. Additionally, retroperitoneal fat pad mass was larger in SLNKO mice following high-fat feeding (**Figure 6C**: $P < 0.01$).

SOL mass was not different between genotype, but was greater ($P < 0.05$) following the HFD relative to chow fed controls (**Figure 7A**), while a trend ($P = 0.10$) towards increased EDL mass existed post-HFD (**Figure 7B**). When expressed as a percentage of body mass, the EDL (**Figure 7E**) of high-fat fed mice was lower relative to chow fed controls (main effect of HFD: $P < 0.001$), while a trend ($P = 0.08$) towards a lower percentage SOL mass (**Figure 7D**) existed post-HFD; no differences were found between genotype. Liver mass of high-fat fed mice was larger relative to chow-fed counterparts, with SLNKO mice having larger livers post-HFD relative to WT controls (**Figure 7C**; $P < 0.01$). When expressed as a percentage of body mass (**Figure 7F**), livers of high fat fed animals comprised a larger percentage of body mass (main effect of HFD: $P < 0.001$). There was a trend ($P = 0.07$) towards a dietary by genotype interaction, with high-fat fed SLNKO mice having a greater % liver than WT counterparts.

Correlational analysis revealed no relationship between total distance run and adiposity index for both high-fat fed WT and SLNKO mice (**Figure 7A and B**); thus, grouped data were used to determine the effects of exercise and high-fat feeding on obesity. Voluntary wheel running did not reduce adiposity in either genotype (**Figure 9**), with SLNKO mice being more obese than WT animals regardless of access to running wheels (**Figure 9**; main effect of genotype: $P < 0.05$).

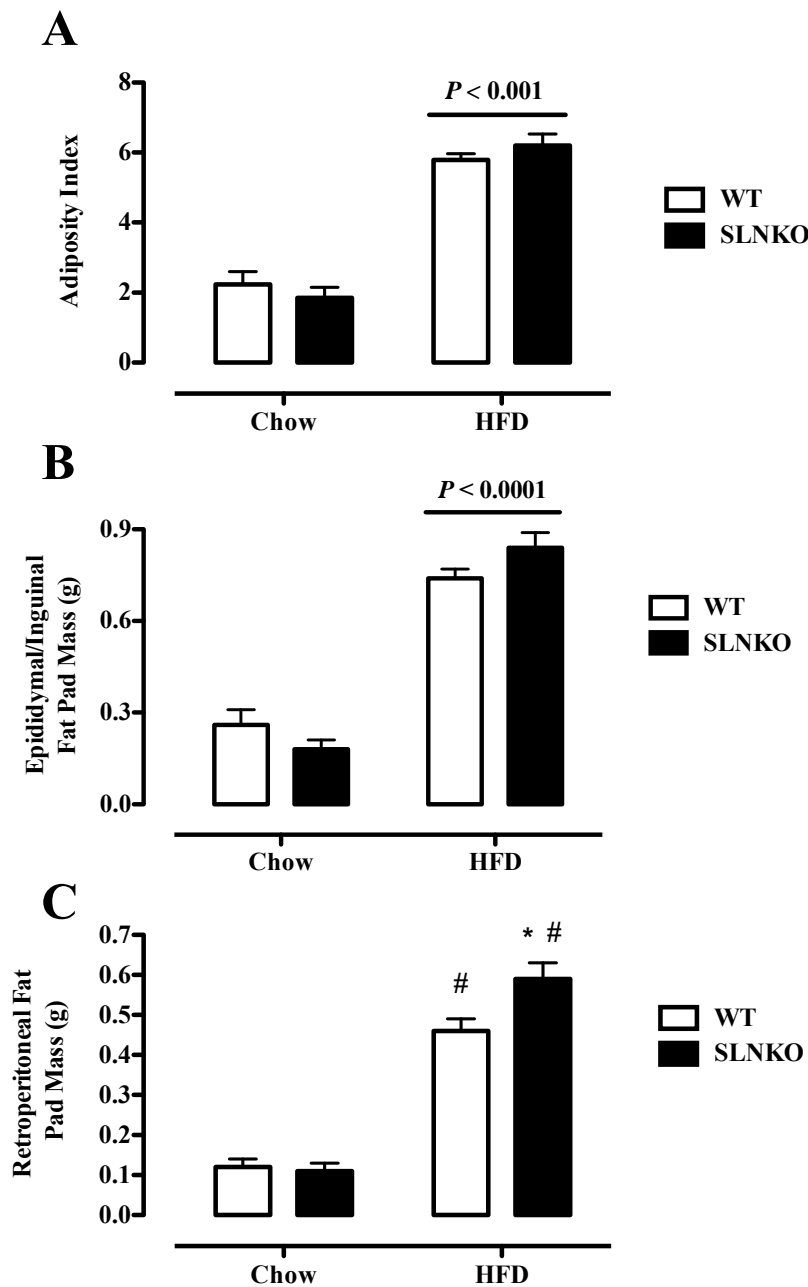


Figure 6. Adiposity measures of sedentary wild-type (WT) and sarcolipin knock-out (SLNKO) mice following 8 weeks of a chow or a high-fat diet (HFD). A) Adiposity index (see methods for formula). A significant main effect of diet existed ($P < 0.001$), with HFD > Chow. B) Epididymal and inguinal fat pad mass (g). A significant main effect of diet existed ($P < 0.0001$), with HFD > chow. C) Retroperitoneal fat pad mass (g). * Significantly different than corresponding WT ($P < 0.01$). # Significantly different than corresponding chow ($P < 0.01$). (WT Chow: $n = 12$, SLNKO Chow: $n = 7$, WT HFD: $n = 9$, SLNKO HFD: $n = 7$). Values are mean \pm S.E.

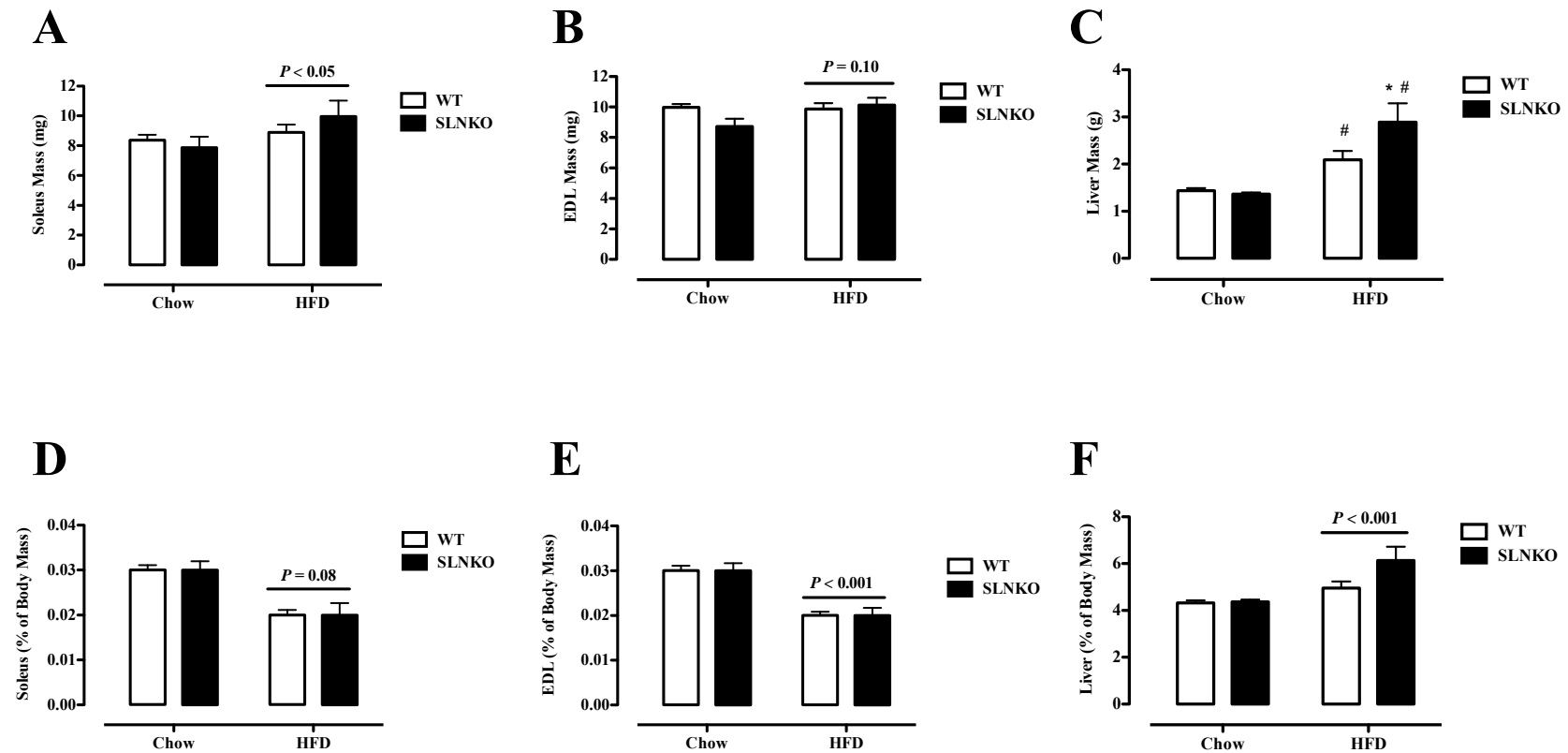


Figure 7. Anthropometric measurements of sedentary wild type (WT) and sarcolipin knock-out (SLNKO) mice following 8 weeks of a chow or high-fat diet (HFD). A) Soleus mass (mg). A significant main effect of diet existed ($P < 0.05$), with HFD $>$ chow. B) Extensor digitorum longus (EDL) mass (mg). C) Liver mass (g). D) Soleus expressed as a percentage of body mass. E) EDL expressed as a percentage of body mass. A significant main effect of diet existed ($P < 0.001$), with HFD $<$ chow. F) Liver expressed as a percentage of body mass. A significant main effect of diet existed ($P < 0.001$), with HFD $>$ chow. (WT Chow: $n = 12$, SLNKO Chow: $n = 7$, WT HFD: $n = 9$, SLNKO HFD: $n = 7$). Values are mean \pm S.E. * Significantly different than corresponding WT ($P < 0.01$). # Significantly different than corresponding chow ($P < 0.05$).

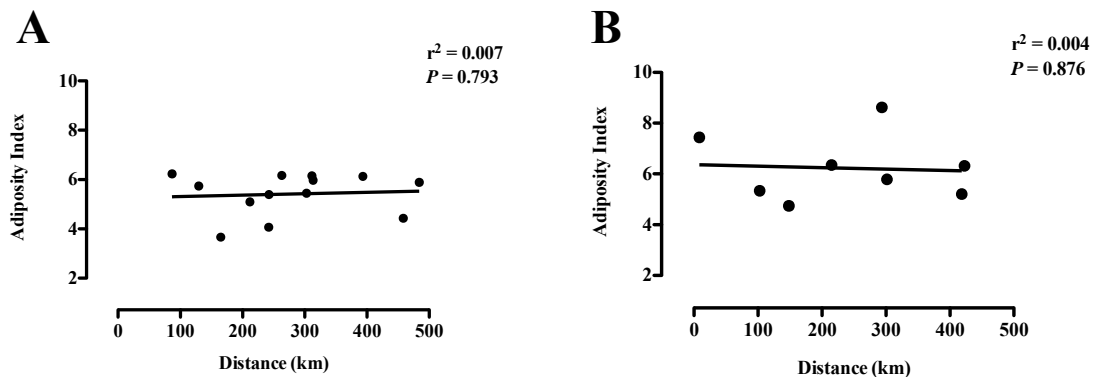


Figure 8. Relationship between total distance run (km) over 8 weeks and adiposity of A) wild type mice (WT; n = 13), and B) sarcolipin knock-out mice (SLNKO; n = 8) following high-fat feeding.

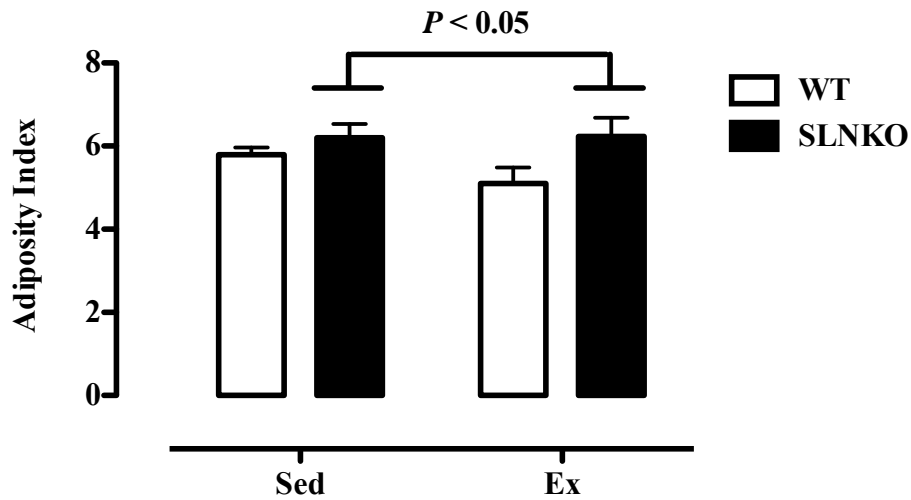


Figure 9. Adiposity index wild type (WT) and sarcolipin knock-out (SLNKO) mice that remained sedentary (Sed) or were given access to voluntary running wheels (Ex) during 8 weeks of high-fat feeding. A significant main effect of genotype existed ($P < 0.05$), with SLNKO $>$ WT. (WT Sed: n = 9, SLNKO Sed: n = 7, WT Ex: n = 13, SLNKO Ex: n = 8). Values are mean \pm S.E.

Glucose Tolerance

Following the HFD in sedentary animals, a trend towards increased fasting blood glucose concentration existed (**Figure 10A**; $P = 0.07$). Additionally, there was a trend ($P = 0.07$) towards a main effect of genotype, with fasting blood glucose being higher in SLNKO mice both pre- and post-HFD (**Figure 10A**). High-fat feeding resulted in impaired whole-body glucose tolerance relative to pre-HFD values in both WT and SLNKO mice (**Figure 10B**; main effect post-HFD: $P < 0.001$), with SLNKO mice being more glucose intolerant relative to WT controls, both pre- and post-HFD (main effect of genotype: $P < 0.05$). Planned comparisons of post-HFD glucose tolerance between genotype (2-way repeated measures ANOVA) revealed a trend ($P = 0.10$) toward higher blood glucose in SLNKO mice, but this was not found between genotype for pre-diet measures. When expressed as AUC, glucose tolerance was impaired following the HFD in both genotypes (**Figure 10C**; main effect post-HFD: $P < 0.001$), again with AUC values of SLNKO mice greater pre- and post-HFD relative to WT counterparts (main effect of genotype: $P < 0.05$). Planned comparisons (Student's 1-tailed t-test) revealed a trend ($P = 0.09$) towards greater AUC in SLNKO mice only post-HFD.

Similar to sedentary animals, mice given access to voluntary running wheels during a HFD had higher post-diet fasting blood glucose compared to pre-diet measurements (**Figure 11A**; main effect post- HFD: $P < 0.05$), but no differences were seen between genotype. High-fat feeding resulted in impairment of glucose tolerance in both WT and SLNKO mice relative to their respective pre-dietary values (**Figure 11B**; $P < 0.0001$); however, no genotype difference existed post-HFD. Planned comparisons of post-HFD glucose tolerance measurements (2-way repeated-measures ANOVA) also

revealed no differences between SLNKO and WT mice. When expressed as AUC, post-HFD glucose tolerance was impaired relative to pre-diet measurements in both WT and SLNKO mice (**Figure 11C**; main effect post-HFD: $P < 0.0001$). Planned comparisons (Student's 1-tailed t-test) between genotype revealed that post-HFD AUC was greater ($P < 0.05$) in SLNKO mice compared to WT counterparts.

To determine the effect of activity on glucose handling following high-fat feeding, post-diet glucose tolerance tests were compared between sedentary and exercising mice. No effect of exercise on glucose tolerance existed for both WT and SLNKO mice (**Figure 12A and B**), while SLNKO mice were more glucose intolerant relative to WT controls, regardless of activity status (main effect of genotype: $P < 0.05$). No relationship between total distance run and post-HFD glucose tolerance (expressed as AUC) existed for WT mice (**Figure 13A**); however, a significant negative relationship was found in SLNKO mice (**Figure 13B**; $P < 0.05$, $r^2 = 0.691$), with lower glucose intolerance seen in SLNKO mice that completed a greater volume of exercise.

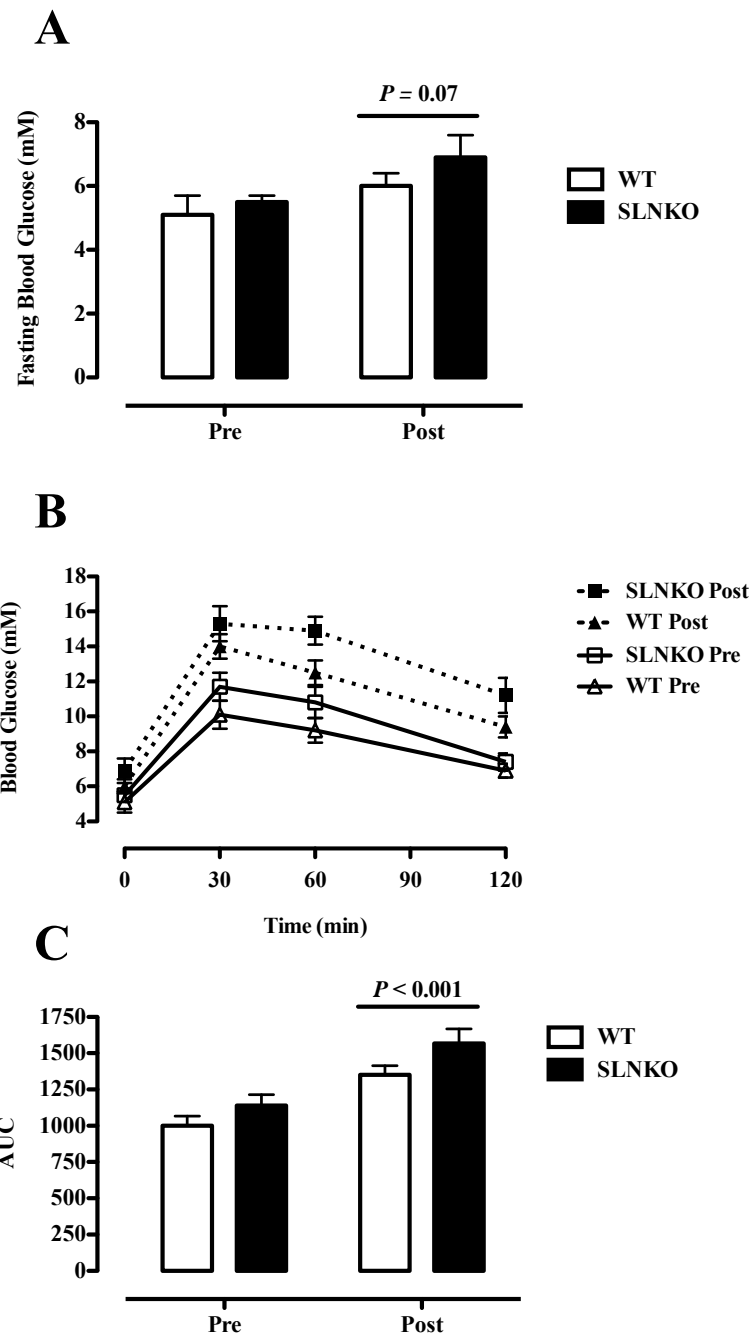


Figure 10. Glucose handling of sedentary wild-type (WT; n = 8) and sarcolipin knock-out (SLNKO; n = 8) mice before (Pre) and after (Post) a high-fat diet. A) Pre- and post-diet fasting blood glucose (mM). A significant main effect of pre/post existed ($P < 0.05$), with post > pre. B) Pre- and post-diet whole-body glucose tolerance test. A significant main effect of time ($P < 0.0001$) and pre/post ($P < 0.0001$) existed, with post > pre values. C) Glucose tolerance pre and post-diet expressed as AUC. A significant main effect of time existed ($P < 0.001$), with post > pre. Values are mean \pm S.E.

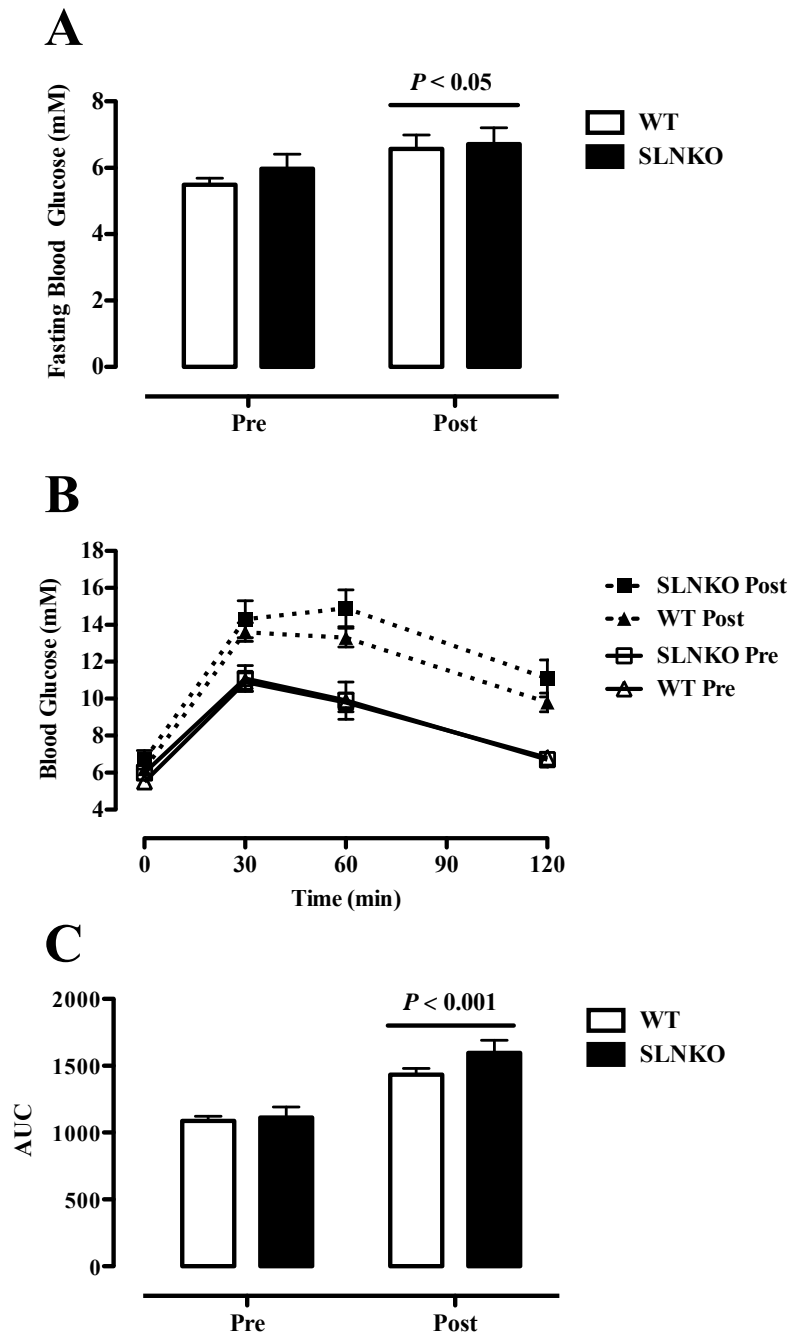


Figure 11. Glucose handling of wild type (WT; $n = 13$) and sarcolipin knock-out (SLNKO; $n = 7$) mice given access to voluntary running wheels and a high-fat diet. **A**) Pre- and post-diet fasting blood glucose (mM). A significant main effect of pre/post existed ($P < 0.05$), with post $>$ pre. **B**) Pre- and post-diet glucose tolerance test. A significant main effect of time existed ($P < 0.0001$), with post $>$ pre. **C**) Pre- and post-HFD glucose tolerance expressed as area under the curve (AUC). A significant main effect of pre/post existed ($P < 0.001$), with post $>$ pre. Values are mean \pm S.E.

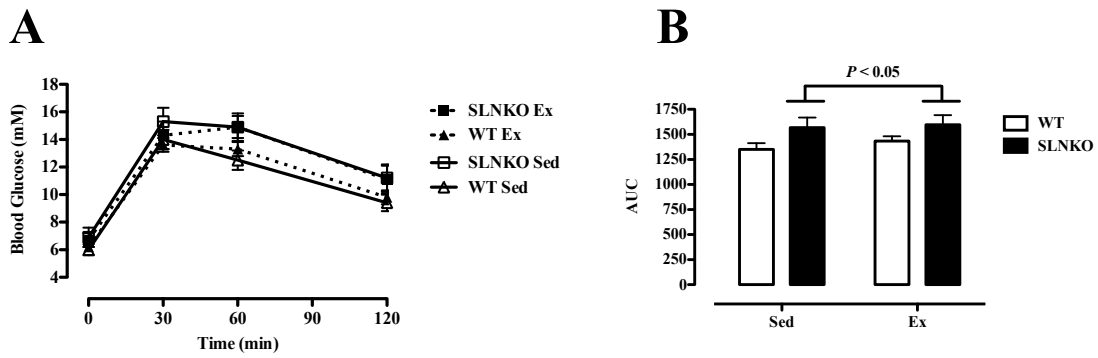


Figure 12. Post-high fat diet (HFD) glucose handling of sedentary (Sed) and voluntary running (Ex) wild type (WT) and sarcolipin knock-out (SLNKO) mice. **A)** Glucose tolerance test. A significant main effect of time ($P < 0.001$) and genotype ($P < 0.05$) existed, with SLNKO $>$ WT. **B)** Post-HFD glucose tolerance expressed as area under the curve (AUC) of Sed and Ex animals. A significant main effect of genotype existed ($P < 0.05$), with SLNKO $>$ WT. (WT Sed: $n = 8$, SLNKO Sed: $n = 8$, WT Ex: $n = 13$, SLNKO Ex: $n = 7$). Values are mean \pm S.E.

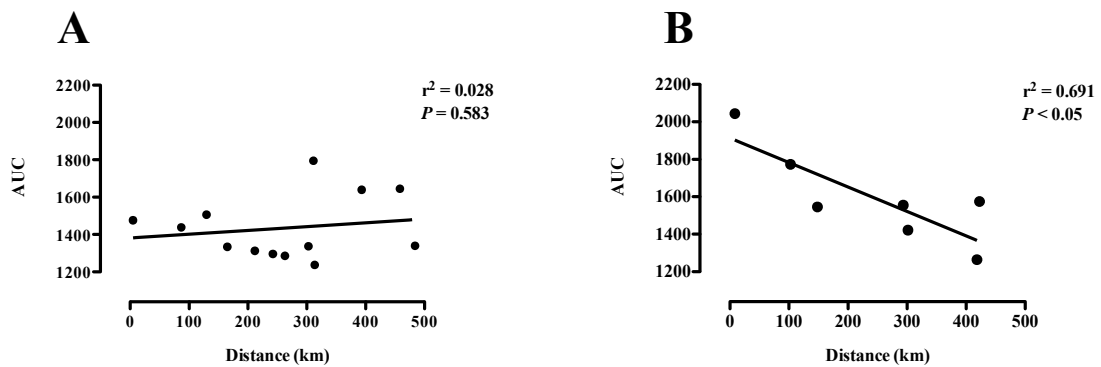


Figure 13. Relationship between total distance run (km) over 8 weeks and glucose tolerance, expressed as area under the curve (AUC), for high fat fed **A)** wild type ($n = 13$) and, **B)** sarcolipin knock-out mice (SLNKO: $n = 7$). A significant negative relationship ($P < 0.05$) was observed in SLNKO mice.

CLAMS Measurements

Pre-dietary CLAMS measurements of all study mice are listed in **Table 1**. No differences in body mass, average $\dot{V}O_2$, or food intake were found between genotype. However, total activity and dual beam activity were significantly lower ($P < 0.05$) in SLNKO mice, while a trend ($P = 0.07$) towards a lower RER in SLNKO mice also existed.

Table 1. Baseline CLAMS measurements of wild type (WT: n = 47) and sarcolipin knock-out (SLNKO: n = 31) mice. Values are mean \pm S.E.

	WT	SLNKO
Body Mass (g)	32.3 \pm 0.44	32.0 \pm 0.57
Total $\dot{V}O_2$ (ml/kg/hr)	3037 \pm 41.33	2966 \pm 55.52
Food Intake (g)	4.6 \pm 0.13	4.3 \pm 0.20
Total Activity	11205 \pm 361.44	9916 \pm 292.68*
Dual Beam Activity	3330 \pm 163.61	2842 \pm 117.37*
Total RER	0.97 \pm 0.004	0.95 \pm 0.008

RER: respiratory exchange ratio

* significantly different than WT ($P < 0.05$)

Table 2 contains comparisons of post-HFD CLAMS variables for sedentary and voluntary exercising animals. No differences in body mass were found between genotype, regardless of exercise status; however, planned comparisons between sedentary animals (Student's 1-tailed t-test) indicated a trend ($P = 0.06$) towards greater body mass in SLNKO mice. No differences between genotype were observed in total $\dot{V}O_2$, food intake, total activity, or dual beam activity. Although not significant, total RER tended ($P = 0.07$) to be lower in SLNKO mice, regardless of activity status.

It should be noted that post-HFD total $\dot{V}O_2$, food intake, and total RER (**Table 2**) were all lower ($P < 0.05$) than their respective pre-diet measurements (**Table 1**), regardless of genotype.

Skeletal Muscle Metabolic Enzyme Activities

Table 3 contains SOL and EDL metabolic enzyme activities from sedentary chow-fed mice. In the SOL, no differences in the activities of HEX, PFK, SDH, CS, COX and β -HAD existed between genotype. However, LDH activity in SOL was significantly higher ($P < 0.05$) in SLNKO mice compared with WT controls. In the EDL, no genotype differences in the activities of HEX, PFK, LDH, CS, or β -HAD existed, whereas COX activity was significantly higher ($P < 0.001$) in SLNKO relative to WT mice.

Table 2. Post-diet CLAMS measurements of sedentary (Sed) and voluntary running (Ex) wild type (WT) and sarcolipin knock-out (SLNKO) mice fed a high-fat diet (HFD). (WT Sed: n = 9; SLNKO Sed: n = 8; WT Ex: n = 14; SLNKO Ex: n = 8). Values are mean \pm S.E.

	Post-HFD Sed		Post-HFD Ex	
	WT	SLNKO	WT	SLNKO
Body Mass (g)	44.7 \pm 2.00	49.4 \pm 2.02	45.2 \pm 1.27	46.0 \pm 2.41
Total $\dot{V}O_2$ (ml/kg/hr)	2585 \pm 76.5	2512 \pm 98.0	2606 \pm 70.1	2466 \pm 99.8
Food Intake (g)	3.19 \pm 0.15	2.64 \pm 0.28	2.85 \pm 0.24	2.85 \pm 0.18
Total Activity	9151 \pm 664	9334 \pm 385	9699 \pm 601	8772 \pm 486
Dual Beam Activity	2980 \pm 268	2972 \pm 226	3290 \pm 303	2421 \pm 217
Total RER	0.89 \pm 0.010	0.86 \pm 0.021	0.88 \pm 0.011	0.87 \pm 0.012

RER: respiratory exchange ratio

Within the SOL, high-fat feeding did not alter HEX or PFK activity in WT or SLNKO mice (**Figure 14A and B**), but did decrease ($P < 0.05$) LDH activity by ~18% in SLNKO mice relative to chow-fed counterparts (**Figure 14C**). In the EDL, high-fat feeding resulted in a trend ($P = 0.07$) towards greater HEX activity, but no genotype differences existed (**Figure 14D**). PFK activity was elevated ($P < 0.05$) following the HFD in SLNKO mice, but not in WT counterparts (**Figure 14E**). Lastly, LDH activity within the EDL was greater ($P < 0.05$) in high fat-fed mice relative to chow fed animals, but there were no differences between WT and SLNKO mice (**Figure 14F**).

Mitochondrial enzyme activities within the SOL were unaltered by genotype or diet (**Figure 15A-D**). Similarly, both SDH (**Figure 16A**) and CS (**Figure 16B**) activities within the EDL were not altered by genotype or diet; however, COX activity within the EDL was ~25% greater in SLNKO mice ($P < 0.001$), regardless of diet (**Figure 16C**). Lastly, β -HAD activity was ~30% higher in high-fat fed animals ($P < 0.05$) relative to chow-fed controls (**Figure 16D**), but no differences in activity were seen between genotype.

Table 3. Enzyme activities of the soleus (SOL) and extensor digitorum longus (EDL) in sedentary chow-fed wild type (WT) and sarcolipin knock-out (SLNKO) mice. Activities are expressed as mean \pm S.E. (n). HEX: hexokinase, PFK: phosphofructokinase, LDH: lactate dehydrogenase, SDH: succinate dehydrogenase, CS: citrate synthase, β -HAD: β -hydroxyacyl-CoA dehydrogenase, COX: cytochrome c oxidase.

Enzyme	SOL		EDL	
	WT	SLNKO	WT	SLNKO
HEX	0.31 \pm 0.02 (11)	0.26 \pm 0.03 (7)	0.09 \pm 0.005 (9)	0.09 \pm 0.005 (7)
PFK	10.8 \pm 1.07 (12)	8.25 \pm 0.36 (6)	9.3 \pm 0.59 (11)	9.4 \pm 0.69 (7)
LDH	50.6 \pm 1.16 (11)	60.0 \pm 3.74* (7)	99.2 \pm 2.34 (11)	98.3 \pm 5.29 (7)
SDH	3.88 \pm 0.13 (11)	3.94 \pm 0.23 (7)	2.8 \pm 0.10 (11)	3.0 \pm 0.35 (7)
CS	27.5 \pm 1.65 (11)	32.4 \pm 2.85 (7)	15.9 \pm 1.50 (11)	13.9 \pm 2.23 (7)
COX	1.27 \pm 0.03 (10)	1.27 \pm 0.03 (7)	1.0 \pm 0.04 (11)	1.3 \pm 0.09* (7)
β-HAD	6.45 \pm 0.78 (10)	6.70 \pm 0.73 (7)	1.5 \pm 0.13 (11)	1.4 \pm 0.14 (7)

* significantly different than corresponding WT (P < 0.05)

units: mmol/hr/g protein

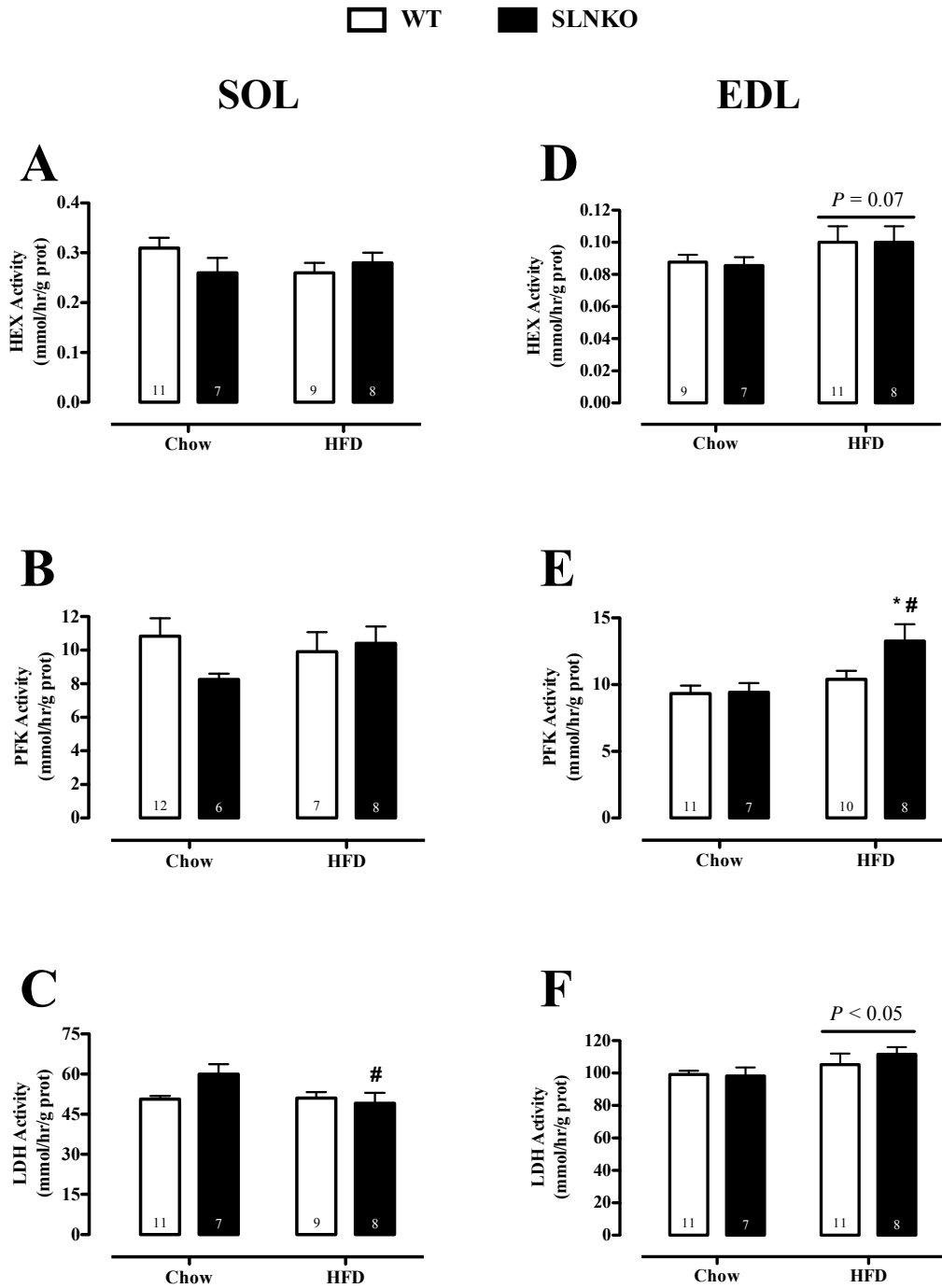


Figure 14. Cytosolic enzyme activities (mmol/hr/g protein) within the soleus (SOL) and extensor digitorum longus (EDL) of sedentary wild type (WT) and sarcolipin knock-out (SLNKO) mice following a chow or high fat diet (HFD). **A)** SOL hexokinase (HEX) activity. **B)** SOL phosphofructokinase (PFK) activity. **C)** SOL lactate dehydrogenase (LDH) activity. **D)** EDL HEX activity. **E)** EDL PFK activity. **F)** EDL LDH activity. A significant main effect post-HFD existed ($P < 0.05$), with HFD > chow. Sample sizes for each group are indicated at the bottom of each bar. * Significantly different than corresponding WT ($P < 0.05$). # Significantly different than corresponding chow ($P < 0.05$). Values are mean \pm S.E.

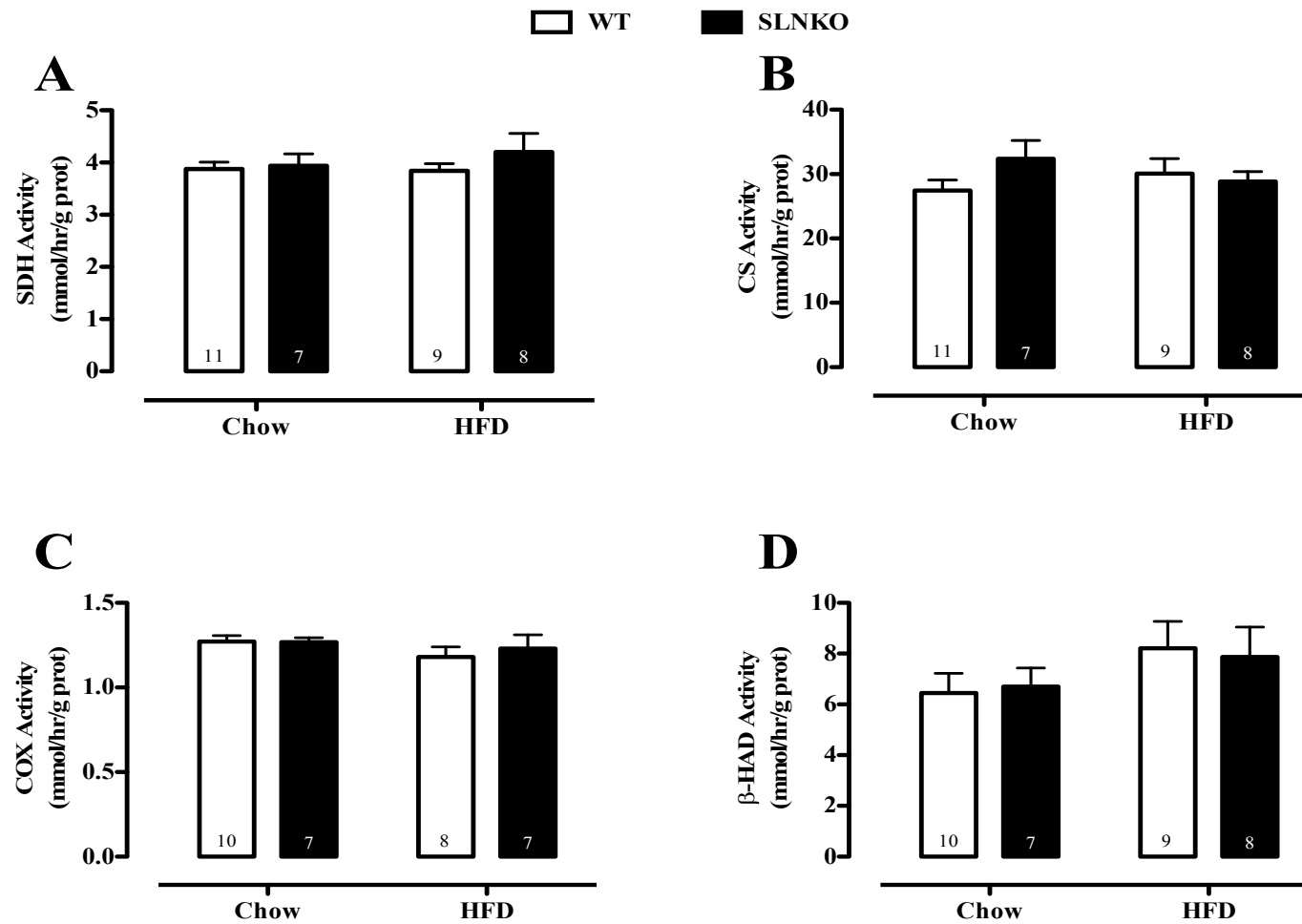


Figure 15. Soleus mitochondrial enzyme activities (mmol/hr/g protein) of sedentary wild-type (WT) and sarcolipin knock-out (SLNKO) mice following a chow or high fat diet (HFD). A) Succinate dehydrogenase (SDH) activity. B) Citrate synthase (CS) activity. C) Cytochrome *c* oxidase (COX) activity. D) β -hydroxyacyl-CoA dehydrogenase (β -HAD) activity. Sample sizes for each group are indicated at the bottom of each bar. Values are mean \pm S.E.

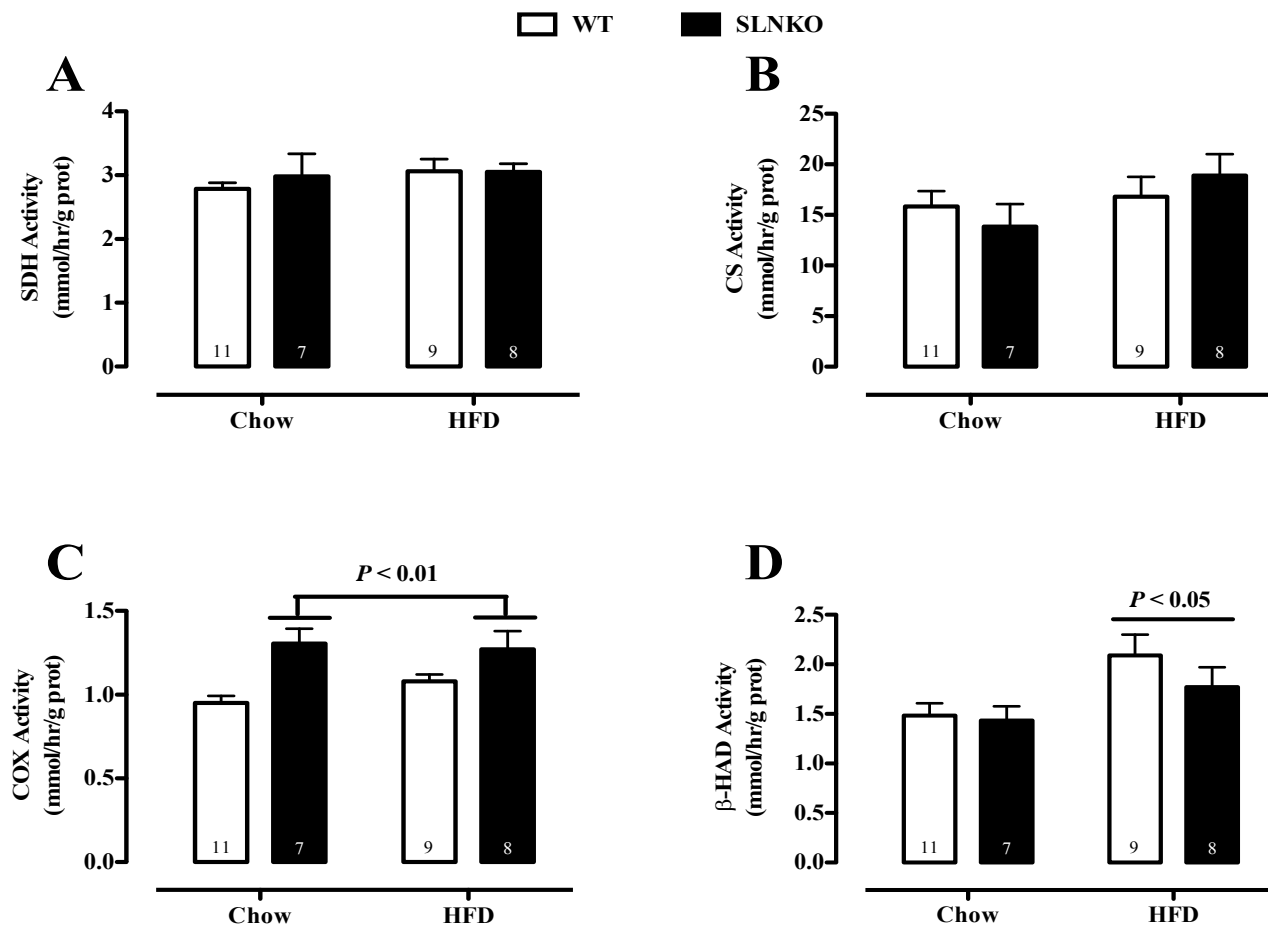


Figure 16. Mitochondrial enzyme activities (mmol/hr/g protein) of the extensor digitorum longus of wild-type (WT) and sarcolipin knock-out (SLNKO) mice following a chow or high fat diet (HFD). **A)** Succinate dehydrogenase (SDH) activity. **B)** Citrate synthase (CS) activity. **C)** Cytochrome *c* oxidase (COX) activity. A significant main effect of genotype existed ($P < 0.001$), with SLNKO > WT mice. **D)** β -hydroxyacyl-CoA dehydrogenase (β -HAD) activity. A significant main effect of diet existed ($P < 0.05$), with HFD > chow. Sample sizes for each group are indicated at the bottom of each bar. Values are mean \pm S.E.

Discussion

This thesis has yielded several novel insights into the SLNKO model of diet-induced obesity. First, as hypothesized, SLNKO mice were more obese and glucose intolerant than WT counterparts following the HFD despite access to and completion of voluntary exercise (**Figure 9 and 12**). In fact, unexpectedly, no relationship was found between exercise volume and adiposity (**Figure 8**), regardless of genotype. Second, no metabolic pre-disposition towards lower skeletal muscle glycolytic or oxidative capacity existed in chow-fed SLNKO mice (**Table 3**). Interestingly, SLN ablation resulted in greater respiratory chain capacity within the EDL (**Figure 16C**), a muscle with low endogenous SLN expression (Tupling *et al.*, 2011). Together, these findings lend further support to SLN's thermogenic role as the primary mechanism of diet-induced obesity in the SLNKO model, not a reduction in skeletal muscle oxidative capacity.

The initial observation that ablation of SLN results in more efficient SR Ca^{2+} -pumping by SERCA led to the hypothesis that SLN has a major function in adaptive thermogenesis, the resultant loss of which predisposes mice to an excessively obese phenotype under conditions of high-fat feeding (Bombardier, 2010). It was of interest to determine whether increasing daily energy expenditure through voluntary exercise could compensate for this loss during caloric excess, especially given that some transgenic models of obesity only display a disease phenotype when chronically sedentary (Huszar *et al.*, 1997; Haskell-Luevano *et al.*, 2009). Daily average running and total exercise volume over 8 weeks were similar between WT and SLNKO mice (**Figure 3**). Similar to other studies in mice (Davidson *et al.*, 2006; Ikeda *et al.*, 2006), running activity increased after week 1 and was maintained fairly constant thereafter. Average daily running distance of WT and SLNKO mice was similar to previous reports (Lightfoot *et al.*, 2004; Davidson *et al.*, 2006; Meek *et al.*, 2009); however, several studies have shown higher mean

daily running distances for male C57BL/6J mice (Lerman *et al.*, 2002; Waters *et al.*, 2004; De Bono *et al.*, 2005). Regardless, daily running activity observed here is within the same order of magnitude as previously shown.

Divergence in mass gain between genotype of sedentary mice only occurred under conditions of high-fat feeding (**Figure 4A**), the magnitude of which was similar to that seen by Bombardier (2010). Several studies have demonstrated comparable changes in mass using similar high-fat diets (Li *et al.*, 2000; Collins *et al.*, 2004). As expected, SLNKO mice gained more mass over the course of 8 weeks relative to WT mice; this is consistent with SLN's thermogenic role, as more ingested calories will be needed by WT mice to maintain skeletal muscle resting Ca^{2+} homeostasis, leaving less available for storage. When mice were given voluntary running access during the HFD, mass gain was similar between genotype (**Figure 4B**). Additionally, voluntary wheel running did attenuate mass gain relative to sedentary controls (**Figure 5**). This is consistent with several studies showing a reduction in dietary mass gain with voluntary exercise and high-fat feeding in both mouse (Bell *et al.*, 1995; Bradley *et al.*, 2008; Vieira *et al.*, 2009) and rat (Podolin *et al.*, 1999). This exercise effect appears to be driven mainly by weight loss in SLNKO mice. Rodents given access to voluntary exercise often compensate for increases in energy expenditure by increasing caloric intake (Garland *et al.*, 2011). Thus, hyperphagia may be an important compensatory control for the maintenance of body mass in WT mice, while activity thermogenesis may be more important in SLNKO mice for preventing excessive mass gain. However, it should be noted that running activity was quite variable within each genotype. As yet, it remains unclear whether the lack of a difference in mass gain of voluntary exercising animals would persist when matched for activity and food intake.

Excessive mass gain of sedentary mice with high-fat feeding resulted in a marked increase in adiposity (**Figure 6A**). Accompanying this increase was an expansion of visceral adipose depots (**Figures 6B and C**) and a concomitant increase in skeletal muscle mass (**Figures 7A and B**). Thus, the increased obesity of sedentary mice likely reflects an increase in fat mass, not a decrease in lean body mass. However, unlike previously reported (Bombardier, 2010), no difference in post-HFD adiposity was observed between WT and SLNKO mice; this lack of a difference could result from the greater post-diet body mass of SLNKO mice (**Table 2**). It should also be noted that the calculated adiposity index is a crude measure of relative body composition as it only incorporates select visceral fat pads. Analysis of fat content through more powerful means, such as dual-energy x-ray absorptiometry, may provide greater discriminating power in whole-body adiposity. Although not statistically significant, SLNKO mice were ~7% more obese than WT counterparts following the HFD (1-tailed t-test: $P = 0.13$). Bombardier (2010) observed ~24% higher adiposity in SLNKO mice following high-fat feeding. Despite this, visceral adipose depots (**Figure 6B and C**) and absolute liver mass (**Figure 7C**) were larger post-HFD in SLNKO animals. These results are still consistent with previous findings of increased diet-induced obesity with SLN ablation (Bombardier, 2010). Expansion of adipose tissue is a common feature in rodent models of diet-induced obesity (West and York, 1998). Increased visceral fat content is believed to lead to the development of metabolic syndrome through “spill-over” of lipids, resulting in ectopic accumulation of fat in cardiac tissue, skeletal muscle, and liver (Despres and Lemieux, 2006). These findings suggest that SLNKO mice are more susceptible to metabolic derangements stemming from increased visceral adiposity and hepatic steatosis.

Interestingly, although voluntary wheel running did reduce mass gain, most notably in SLNKO mice, no relationship was found between total distance run and adiposity for either genotype (**Figure 8**). Several studies have shown that voluntary exercise reduces excessive fat mass and body fat percentage as a result of high-fat feeding (Bell *et al.*, 1995; Bell *et al.*, 1997; Bradley *et al.*, 2008), but this is not always observed in mice (Jung and Luthin, 2010). As hypothesized, SLNKO mice were more obese despite access to voluntary running (**Figure 9**). These data suggest that while voluntary exercise can reduce absolute body mass, particularly in SLNKO mice, it does not reduce the partitioning of calories into adipose tissue.

Glucose tolerance is unaltered in SLNKO mice under conditions of chow feeding; however, a pronounced impairment results when these animals are given a HFD (Bombardier, 2010). In this study, high-fat feeding induced fasting hyperglycemia in both sedentary and exercising animals (**Figures 11 and 12A**), a common consequence of chronic consumption of a “Westernized” diet (Cordain *et al.*, 2005). As hypothesized, and in accordance with previous findings, excessive glucose intolerance occurred in SLNKO mice following high-fat feeding, regardless of activity status (**Figures 10, 11C, and 12**). Several studies have shown comparable impairments in glucose tolerance using a similar HFD (Li *et al.*, 2000; Xu *et al.*, 2011). While not directly assessed here, the observed impairment in glucose tolerance may reflect diminished skeletal muscle insulin-stimulated glucose uptake (i.e. insulin resistance). Reduced skeletal muscle insulin sensitivity with obesity is believed to be due to elevated plasma FFAs. Direct evidence for this has come from euglycemic-hyperinsulinemic clamp studies with concomitant lipid infusion, which show a reduction in peripheral insulin sensitivity with physiological elevations in plasma FFAs (Ferrannini *et al.*, 1983; Boden and Jadali, 1991; Boden and Chen, 1995; Roden *et al.* 1996; Kashyap *et al.*, 2003). SLNKO mice show an exaggerated increase in

both serum non-esterified fatty acids and LDL-cholesterol after high-fat feeding (Bombardier, 2010). The resultant uptake of circulating FFAs by skeletal muscle would be expected to result in the accretion of several lipid metabolites shown to alter insulin signaling, including long-chain fatty acyl-CoAs (Cooney *et al.*, 2002), diacylglycerols (Itani *et al.*, 2002), and ceramides (Chavez *et al.*, 2003). Activation of protein kinase C (PKC) by diacylglycerols reduces insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation, preventing IRS-1 interaction with the insulin receptor (Samuel *et al.*, 2010), while ceramides have been shown to inhibit Akt (Summers, 2006). The resultant action of these lipid metabolites is preventing the translocation of GLUT-4 containing vesicles to the sarcolemma, reducing glucose uptake.

While excessive insulin resistance in SLNKO mice has not been established using the intraperitoneal insulin tolerance test (Bombardier, 2010), this technique does not assess tissue specific insulin sensitivity and may be confounded by counter-regulatory homeostatic mechanisms (Muniyappa *et al.*, 2008). To circumvent these complications, future analysis of insulin sensitivity in these animals should be assessed by euglycemic-hyperinsulinemic clamp, as this is considered the “gold-standard” measure of insulin-sensitivity (Kim, 2009).

Interestingly, no improvement of glucose tolerance occurred with activity (**Figure 12**), suggesting that the amount of exercise engaged in by the experimental animals was inadequate for improvements in glucose handling. Voluntary exercise does improve glucose handling in rodent models of obesity, including Zucker diabetic fatty rats (Kiryaly *et al.*, 2010; Delghingaro-Augusto *et al.*, 2011) and Osborne-Mendel rats (Zachwieja *et al.*, 1997). However, it should be noted that obesity in these models occurs while consuming standard rodent chow. As discussed above, macronutrient composition can have marked effects on glucose handling and insulin sensitivity, despite increased cage activity. Consistent with the findings of this study, Ma and

colleagues (2010) found no improvement in glucose tolerance of voluntary exercising male C57BL/6J mice when fed a HFD. Given that both adiposity and glucose tolerance are not improved in either genotype, the energetic cost of voluntary exercise completed by the study animals is not great enough to compensate for the HFD. However, this is not to say exercise cannot improve diet-induced complication in glucose handling. Xu and colleagues (2011) showed that forced training of high-fat fed male C57BL/6J mice resulted in improvements of glucose tolerance relative to sedentary counterparts. In fact, while glucose tolerance showed no relationship with exercise volume in WT animals, a modest improvement with exercise was seen in SLNKO mice (**Figure 13**).

Improvement in glucose tolerance of SLNKO mice following voluntary exercise may be due to suppression of the adrenergic response to the HFD by activity. Following high-fat feeding, serum epinephrine and norepinephrine are elevated in SLNKO mice (Bombardier, 2010). Rising catecholamines stimulate adipose tissue and skeletal muscle lipolysis (Jocken and Blaak, 2008), and increases BAT mitochondrial uncoupling (Collins and Surwitt, 2001), both of which are adaptive responses to caloric excess. Although this coordinated adrenergic response will increase energy expenditure and lipid utilization, several studies have shown that epinephrine inhibits skeletal muscle glucose uptake (Lee *et al.*, 1997; Hunt and Ivy, 2002). However, a common adaptation to physical training is a reduction in the concentration of circulating catecholamines during exercise (Duncker and Bache, 2008). Given that adiposity was greater with SLN ablation, it is conceivable that a reduction in circulating catecholamines with voluntary exercise is at least partly responsible for the modest improvements in glucose tolerance seen in SLNKO mice. In accordance with this, β -adrenergic blockade with propranolol improves glucose tolerance of sedentary high-fat fed SLNKO mice (unpublished data).

However, serum catecholamines of exercising mice were not measured in this study; future analysis should examine this adrenergic response to voluntary exercise.

The relationship between SERCA and SLN is thermogenic in nature (Smith *et al.*, 2002; Mall *et al.*, 2006; Bombardier, 2010). Given that ablation of SLN reduces the energy requirement of SERCA Ca^{2+} pumping, one would expect whole-body metabolic rate to be lower in SLNKO mice. However, no difference in $\dot{V}O_2$ was seen between chow-fed WT and SLNKO animals (**Table 1**). This is consistent with previous findings showing no difference in metabolic rate with SLN ablation (Bombardier, 2010). Given that energy expenditure was similar between genotype, it is not surprising that both body mass and food intake also did not differ. Increased cage activity cannot explain this lack of difference in whole-body metabolism, as SLNKO mice were less active during the sampling period. Instead, thermogenic compensation likely occurs by other means, namely through UCP-3 expression, which is elevated in the SOL of SLNKO mice (Bombardier, 2010). It cannot be ruled out that other “energy wasting” processes also contribute to the maintenance of metabolic rate with SLN ablation, including increased protein turnover and leakage of other ions (i.e. Na^+ , K^+) requiring active transport (Lowell and Spiegelman, 2000).

Despite SLNKO mice developing an excessively obese phenotype with high-fat feeding, no detectable difference in post-diet metabolic rate was observed between genotype, regardless of activity status (**Table 2**). Previous findings show that decreases in whole-body metabolic rate with high-fat feeding is attenuated in WT mice, due in part to an increase in skeletal muscle SLN expression (Bombardier, 2010). Thus, a diet-induced reduction of SERCA pumping efficiency protects WT mice from excessive obesity. It is possible that SLN expression in this cohort of WT mice did not increase to levels previously seen, resulting in no detectable difference in whole-body metabolic rate between genotype. However, this lack of difference in post-HFD

$\dot{V}O_2$ can explain why the genotype disparity in adiposity and glucose tolerance were not as great as that shown by Bombardier (2010). Although not significant, post-HFD $\dot{V}O_2$ was ~4% lower in SLNKO animals; even a slight reduction in metabolic rate could result in detectable weight gain over a prolonged period of time, which was seen here. Thus, the post-diet measurement period may not accurately reflect changes in metabolic rate that occur during the 8 weeks of high-fat feeding. Additionally, no effect of voluntary running access on $\dot{V}O_2$ was observed. This finding is not surprising, as voluntary wheel running in rodents does not result in an increase in resting metabolic rate (Speakman and Selman, 2003). Although voluntary exercise did not increase $\dot{V}O_2$, exercising mice, particularly SLNKO, gained less mass. This is likely due to the increased energy demand of voluntary running itself, and not a training induced increase in basal metabolism. Even though whole-body metabolism was similar, it was also of interest to examine specific pathways of energy metabolism within skeletal muscle, as this may help explain the susceptibility to and development of obesity with SLN ablation.

Obesity is associated with a decrease in skeletal muscle mitochondrial content (Kelley *et al.*, 2002; Boushel *et al.*, 2007; Holloway *et al.*, 2007). Additionally, individuals with a family history of T2DM show a reduction in the expression of oxidative genes within skeletal muscle (Patti *et al.*, 2003). This has led some to propose that a reduction of oxidative capacity is an initiating factor in the pathogenesis of obesity and T2DM. Given the role cellular Ca^{2+} plays in the signaling of mitochondrial biogenesis (discussed above), examination of several enzymes of energy metabolism in the SLNKO model was conducted. As hypothesized, no changes in mitochondrial enzyme activities in the SOL were seen with SLN ablation (**Table 3**). However, both SOL LDH and EDL COX activities were elevated in SLNKO mice. These findings cannot be explained by changes in fiber-type composition, which is similar between genotype (Tupling

et al., 2011). The finding that COX activity was elevated within the EDL of SLNKO mice is especially surprising given that SLN expression is low within the EDL of WT mice, but nevertheless is detectable at low levels (Tupling *et al.*, 2011). Currently, it is unclear whether increased activity reflects greater protein expression or an increase in the activation state of these enzymes. Additionally, the causal link between SLN ablation and increased enzyme activity in these two tissue beds is unclear, although gene expression of both anaerobic glycolytic and the electron transport chain pathways are, in part, Ca²⁺-dependent (Semenza *et al.*, 1994; Koh *et al.*, 2008, Freyssenet *et al.*, 1999). Regardless, ablation of SLN does not result in a deficit in the capacity of either glycolytic or oxidative energy pathways under chow-fed conditions.

Given that excessive obesity in SLNKO mice only occurs during high-fat feeding, it was logical to examine these same enzymes following caloric excess. Analysis of glycolytic enzymes revealed several changes with high-fat feeding, most of which were exclusive to fast-twitch muscle. Similar to that shown here (Figure 16A), several others have found no alteration of HEX activity in rodent slow-twitch muscle following a HFD (Zierath *et al.*, 1997; Krisan *et al.*, 2004); however, HEX activity was ~11% higher within the EDL (**Figure 14D**). HEX exists in several isoforms (I-IV), of which HEXII predominates in mammalian skeletal muscle (Printz *et al.*, 1997). HEXII gene expression is regulated, in part, by insulin (Printz *et al.*, 1993). Following high-fat feeding, circulating insulin concentration is increased in both WT and SLNKO mice (Bombardier, 2010). Increased HEX activity observed here likely reflects activation by diet-induced hyperinsulinemia. Interestingly, PFK activity was elevated post-HFD in the EDL of SLNKO mice only, while no change was observed within the SOL (**Figure 14B and E**). As mentioned above, circulating catecholamines are elevated in SLNKO mice post-HFD. Given that epinephrine activates skeletal muscle PFK activity (Alves and Sola-Penna,

2003), it is not surprising that SLNKO mice show an exaggerated increase in their glycolytic capacity. Lastly, high-fat feeding caused LDH activity within the EDL of both WT and SLNKO mice to increase (**Figure 14F**). The increased LDH activity of high-fat fed mice mimics that of HEX, suggesting obesity results in greater anaerobic capacity within fast-twitch muscle. However, post-HFD RER is lower than that of chow-fed animals, indicating a greater reliance on lipids (**Table 1 and 2**). This apparent divergent response is somewhat confusing, and the mechanism(s) mediating the enhancement of lactate metabolism following the HFD remain to be seen.

Several studies have shown that high-fat feeding results in increased oxidative enzyme capacity in rodent skeletal muscle (Hancock *et al.*, 2008; Nemeth *et al.*, 1992; Simi *et al.*, 1991). Interestingly, high-fat feeding did not alter mitochondrial enzyme activities within the SOL, regardless of genotype (**Figure 15**), while all enzymatic adaptations that did occur were specific to fast-twitch muscle (**Figure 16**). No alterations in the activities of SDH (**Figures 15A and 16A**), CS (**Figures 15B and 16B**), or COX (**Figures 15C and 16C**) were observed within the SOL, regardless of diet or genotype. These findings are consistent with those of several others who show no change in the activities of citric acid cycle or electron transport chain markers with high-fat feeding in rodent slow-, or fast-twitch muscle (Miller *et al.*, 1984; Nemeth *et al.*, 1992; Shreekumar *et al.*, 2002; Sparks *et al.*, 2005; Gupte *et al.*, 2009). It should be noted that, like chow-fed mice, COX activity within the EDL remained elevated in SLNKO animals post-HFD. This gives further conformation that SLN ablation selectively increases electron transport chain capacity of fast-twitch muscle. However, the mechanism(s) by which this occurs remains unknown.

Although not significant, β -HAD activity was ~22% higher in the SOL of high-fat fed mice (**Figure 15D**). An appreciable increase in β -HAD activity was also seen in the EDL post-HFD, and was similar between genotype (**Figure 16D**). Several other studies have shown selective increases in β -oxidative capacity of rodent slow- and fast-twitch muscle without concomitant changes of citric acid cycle markers following diet-induced obesity (Simi *et al.*, 1991; Nemeth *et al.*, 1992). Increased β -oxidative capacity in response to diet is mediated by the nutritionally responsive peroxisome proliferator activated receptors (PPARs). PPARs are endogenous receptors of lipids and lipid metabolites that, along with the “master regulator” of mitochondrial biogenesis PGC-1, organize the transcriptional response of nuclear and mitochondrial genomes in response to diet and exercise (Muio and Koves, 2007). The induction of PPARs/PGC-1 leads to increased transcription of a number of oxidative genes, including those of β -oxidation (Lin *et al.*, 2005).

In light of these findings, high-fat feeding results in a specific adaptation within skeletal muscle to increase the capacity for lipid oxidation only, the response of which is not limited by SLN ablation. However, this is not to say Ca^{2+} -mediated signaling is not altered in SLNKO mice. Given that redundancy exists in biological signaling mechanisms, it is plausible that lack of an overt change in the metabolic phenotype of SLNKO animals is the result of signaling compensation. Regardless, these findings lend further support to SLN’s thermogenic role in the development of excessive diet-induced obesity, and not through an alteration in skeletal muscle metabolism.

In summary, SLNKO mice developed excessive diet-induced obesity and glucose intolerance despite having access to voluntary exercise. This suggests that increasing energy expenditure through activity cannot compensate for increased basal SERCA Ca^{2+} -pumping

efficiency during caloric excess. Additionally, ablation of SLN does not result in a metabolic deficit within skeletal muscle, nor does it limit the adaptive enzymatic response of these mice to high-fat feeding. Together, this study provides further support of SLN's thermogenic role in diet-induced obesity, and highlights this protein's important role in skeletal muscle energy expenditure during both rest and activity.

Study Limitations

While some argue that voluntary exercise models help to elucidate the role of a gene/protein in a disease pathogenesis and eliminate problems associated with unrepresentative levels of physical inactivity in highly active rodents (Booth and Laye, 2009), they can also complicate interpretation of results. Because experimental animals were given *ad libitum* access to free running wheels, the intensity, time, and duration of voluntary exercise could not be standardized within and between genotype. It is evident from both daily average running distance and total exercise volume that exercise activity is highly variable. Therefore, it is plausible that animals at the extreme ends of completed activity may have skewed some of the whole body measurements (e.g. adiposity and glucose tolerance). Additionally, while mice may have completed a similar volume of exercise (i.e. total distance), the time to complete this amount may have differed substantially; running speed would then impact the amount of muscle recruited during exercise and the energy systems utilized to support this activity, potentially exerting variable whole-body effects. This may explain why voluntary exercise reduced mass gain in high-fat fed animals, while paradoxically, had no effect on adiposity.

Additionally, due to the experimental design, individuals from each treatment group were housed together in the same room at any given time. While this makes sense to control for the

effects of time, it may have been problematic due to continuous noise made by the running wheels. This may have elicited a greater stress response in surrounding littermates.

Furthermore, although sedentary mice had a locked running wheel placed in their cage, it cannot be ruled out that climbing activity was increased. Both of these factors may have contributed to the lower adiposity disparity seen between genotype, as these were not the exact housing conditions used by Bombardier (2010).

Another limitation of this thesis surrounds the use of enzymatic activity, particularly those within the mitochondria. It has been established in both rodents and humans that CS activity correlates positively with mitochondrial content (Williams *et al.*, 1986; Wang *et al.*, 1999). However, while certain mitochondrial marker enzymes may indirectly reflect mitochondrial abundance, the information they provide may be of little physiological relevance with respect to obesity and T2DM. While skeletal muscle mitochondrial content is reduced in human obesity (Kelley *et al.*, 2002; Boushel *et al.*, 2007; Holloway *et al.*, 2007), the functionality of these remaining mitochondria and their contribution to the disease pathogenesis are a point of contention. Simultaneous examination of marker enzymes of β -oxidation, the citric acid cycle, and electron transport chain may indicate enhancement/impairment of a specific pathway or proportional flux through those pathways (Rabøl *et al.*, 2006), but they are not surrogate measurements of mitochondrial function. Although no reduction in mitochondrial content with SLN ablation was observed here, no inference into their functionality within either genotype can be made from enzymatic measurements alone. Lastly, enzymatic measurements were taken from whole-muscle homogenates; thus, changes in subsarcolemmal and intermyofibrillar mitochondria could not be assessed. It is possible that SLN ablation may have a greater affect on the intermyofibrillar mitochondrial population given their proximity to the SR.

Conclusions

As hypothesized, ablation of SLN resulted in excessive obesity and glucose intolerance following the HFD, regardless if mice remained sedentary or were given access to voluntary exercise. Similar to previous results from our laboratory, sedentary SLNKO mice gained more mass following high-fat feeding than WT counterparts. However, voluntary exercise did attenuate mass gain, most notably in SLNKO animals, but interestingly this was not accompanied by reduced adiposity with wheel running. Additionally, while the HFD resulted in glucose intolerance in both sedentary and exercising animals, voluntary exercise did modestly improve glucose handling in SLNKO mice. While these findings indicate that when paired with a HFD voluntary exercise may attenuate mass gain and induce modest improvement in glucose tolerance, susceptibility to an excessively obese phenotype in SLNKO mice remains. Thus, the increased energy demand of daily ambulation cannot compensate for the reduced energetic cost of SERCA Ca^{2+} -pumping with SLN ablation.

As expected, SLNKO animals were not predisposed to a reduced glycolytic or oxidative capacity within skeletal muscle. Additionally, the development of an excessively obese phenotype in these animals post-HFD was not accompanied by a deficit in the capacity of these enzymatic markers. In accordance with several reports, high-fat feeding resulted in an enhancement of β -oxidative capacity, of which was not limited by SLN ablation. In fact, SLNKO mice showed greater respiratory chain capacity within fast-twitch skeletal muscle. These findings were consistent with a lower RER in SLNKO mice, indicating a greater whole-body utilization of lipids. Taken together, these data suggest that SLN ablation does not negatively affect Ca^{2+} -signaling of energy yielding pathways, and provides further support of the

view that SLN's thermogenic role is the primary mechanism of diet-induced obesity in SLNKO mice.

Future Directions

While voluntary exercise appeared to be ineffective at improving diet-induced obesity and glucose intolerance, further examination with this model is required. Given that running activity was variable, increasing the sample size of exercising mice will improve statistical power and allow for potential matching between genotype based on activity level. This latter point will better indicate whether a genotype difference persists, despite the completion of an equal amount of activity. To circumvent problems associated with voluntary wheel running, utilization of a forced exercise training protocol on a motor driven treadmill would be appropriate. This will allow for the precise control of exercise frequency, duration, and intensity between genotype. At least one study has shown a marked reduction of diet-induced obesity, improved glucose tolerance and insulin sensitivity in male C56BL/6J mice with forced training (Xu *et al.*, 2011).

Future biochemical and molecular analysis should include measurement of enzyme protein expression using Western blotting. Although improvements in β -oxidative capacity are likely the result of greater protein abundance given the length of the HFD, this cannot be conclusively determined using activity alone. It is possible that changes in enzyme activity are due to post-translational modifications, which may be differentially altered in SLNKO mice. Western blotting will be of particular importance for COX, as increased COX activity within the EDL of SLNKO mice was unexpected. The mechanism of improved respiratory chain capacity with SLN ablation is completely unknown from the current study. Thus, Ca^{2+} -dependent

signaling pathways within fast-twitch muscle (i.e. CAMK, calcineurin) require examination to help explain this finding.

While enzymatic activity is reflective of mitochondrial content, debate surrounds the functionality of skeletal muscle mitochondria and its role in the pathogenesis of obesity and T2DM. Future studies of diet-induced obesity in the SLNKO model should utilize [1-¹⁴C] palmitate oxidation in both isolated mitochondria and whole muscle homogenates to determine whether SLN ablation reduces mitochondrial function. This is particularly important, as a number of Ca²⁺-regulated genes coordinate mitochondrial biogenesis and function (Chin, 2004/2005). Given that several important sites involved in oxidative metabolism could limit mitochondrial function, including substrate uptake and catabolism, this technique would eliminate misinterpretation associated with examining oxidative pathways in isolation.

Lastly, utilization of a muscle-specific SLN overexpression model using transgenic mice containing a tetracycline-response element to acutely induce SLN protein expression during high-fat feeding would eliminate complications associated with the global SLNKO model. This would also provide further support for the examination of SLN and PLN within human muscle, and determine whether these Ca²⁺-handling proteins are potential therapeutic targets for obesity and T2DM prevention.

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