

Carnitine Acetyltransferase and Mitochondrial Acetyl-CoA Buffering
in Exercise and Metabolic Disease

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy, in the Department of
Pharmacology and Cancer Biology in the Graduate School
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ABSTRACT

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Abstract

Acetyl-CoA holds a prominent position as the common metabolic intermediate of glucose, amino acid and fatty acid oxidation. Because acetyl-CoA fuels the tricarboxylic acid (TCA) cycle which produces reducing equivalents that drive mitochondrial oxidative phosphorylation, understanding acetyl-CoA pool regulation becomes imperative to understanding mitochondrial energetics. Carnitine acetyltransferase (CrAT), a muscle-enriched mitochondrial enzyme, catalyzes the freely reversible conversion of acetyl-CoA to its membrane permeant carnitine ester, acetylcarnitine. Because CrAT has long been thought to regulate the acetyl-CoA metabolite pool, we investigated the role of CrAT in acetyl-CoA regulation. Although the biochemistry and enzymology of the CrAT reaction has been well studied, its physiological role remains unknown. Investigations herein suggest that CrAT-mediated maintenance of the mitochondrial acetyl-CoA pool is imperative for preservation of energy homeostasis. We provide compelling evidence that CrAT is critical for fine-tuning acetyl-CoA balance during the fasted to fed transition and during exercise. These studies suggest that compromised CrAT activity results in derangements in mitochondrial homeostasis.

In chapter 3, we examined the effects of obesity and lipid exposure on CrAT activity. Recent studies have shown that acetyl-CoA-mediated inhibition of

pyruvate dehydrogenase (PDH), the committed step in glucose oxidation, is modulated by the CrAT enzyme. Because PDH and glucose oxidation are negatively regulated by high fat feeding and obesity, we reasoned that nutritional conditions that promote lipid availability and fat oxidation might likewise compromise CrAT activity. We report an accumulation of long chain acylcarnitines and acyl-CoAs but a decline in the acetylcarnitine/acetyl-CoA ratio in obese and diabetic rodents. This reduction in the skeletal muscle acetylcarnitine/acetyl-CoA ratio was accompanied by a decrease in CrAT specific activity, despite increased protein abundance. Exposure to long chain acyl-CoAs *in vitro* demonstrated that palmitoyl-CoA acts as a mixed model inhibitor of CrAT. Furthermore, primary human skeletal muscle myocytes exposed to fatty acid and or CPT1b overexpression had elevated long chain acylcarnitines but decreased production and efflux of CrAT-derived short chain acylcarnitines. These data suggest that exposure to fatty acids in obesity and diabetes can counter-regulate the CrAT enzyme leading to decreased activity.

Alternatively, chapter 4 addresses the importance of acetyl-CoA buffering during exercise and suggests that a deficit in CrAT activity leads to fatigue. Because CrAT is highly expressed in tissues specifically designed for work and because acetylcarnitine, the primary product of the CrAT reaction, is increased during contraction, we reasoned that CrAT could play an important role in exercise. To investigate this possibility, we employed exercise intervention and

ex-vivo analysis on a genetically novel mouse model of skeletal muscle CrAT deficiency (CrAT^{SM-/-}). Though resting acetyl-CoA levels were elevated in CrAT^{SM-/-} mice, these levels dropped significantly after intense exercise while acetylcarnitine content followed the opposite pattern. This contraction-induced acetyl-CoA deficit in CrAT^{SM-/-} mice was coupled with compromised performance and diminished whole body glucose oxidation during high intensity exercise. These results imply that working muscles clear and consume acetylcarnitine in order to maintain acetyl-CoA buffering during exercise. Importantly, provision of acetylcarnitine enhanced force generation, delayed fatigue and improved mitochondrial energetics in muscles from CrAT^{fl/fl} controls but not CrAT^{SM-/-} littermates, emphasizing the importance of acetyl-CoA pool maintenance. In aggregate, these data demonstrate a critical role for CrAT-mediated acetyl-CoA buffering in exercise tolerance and suggest its involvement in energy metabolism during skeletal muscle contraction and fatigue. These findings could have important clinical implications for individuals with muscle weakness and fatigue due to multiple conditions, such as peripheral vascular or cardiometabolic disease.

In summary, data herein emphasize the role of CrAT in regulation of the mitochondrial acetyl-CoA pool. We demonstrate that CrAT is critical for fine-tuning acetyl-CoA balance both during the fasted to fed transition and during exercise. These data suggest that a deficit in CrAT activity leads to glucose

intolerance and exercise fatigue. We examine these studies and suggest future areas of study.

Dedication

This thesis is dedicated to the memory of my father, Steven Seiler (1953-2002).

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1 Introduction

1.1 *Acetyl-CoA: The Universal Substrate*

Acetyl-CoA holds a predominant position as a key substrate and regulatory metabolite in multiple enzymatic processes. Multiple cellular pools of acetyl-CoA exist. Mitochondrial acetyl-CoA, the two carbon metabolic intermediate of glucose, amino acid and fatty acid oxidation, is a primary substrate of the tricarboxylic acid cycle (TCA cycle). As such, the oxidation of acetyl-CoA fuels the production of reducing equivalents which are required for ATP generation. Alternatively, cytosolic acetyl-CoA acts as a building block for *de novo* fatty acid synthesis and elongation. When citrate synthesis exceeds TCA cycle flux, the resulting buildup of mitochondrial citrate can be exported to the cytosol via the citrate carrier. Citrate is then catabolized to oxaloacetate and acetyl-CoA by citrate lyase and may be utilized for lipogenesis following carboxylation to malonyl-CoA. Therefore, given this dual role as an oxidizable substrate and building block, acetyl-CoA is widely recognized as a universal substrate for both catabolic and anabolic processes.

Acetyl-CoA has a well established role in enzymatic regulation. Mitochondrial acetyl-CoA is an allosteric inhibitor of pyruvate dehydrogenase (PDH), the enzyme complex that couples glycolysis to glucose oxidation. Moreover, acetyl-CoA has been shown to stimulate the activity of PDH kinases, which phosphorylate and inhibit PDH (Sugden and Holness 2006). In addition to

its role as a regulator of PDH activity, acetyl-CoA provides the substrate for acetylation of several key metabolic enzymes. Lysine acetylation, a reversible post-translational modification, has become increasingly recognized as a regulator of enzymatic activity. Supporting this hypothesis, an estimated 35% of mitochondrial proteins have been shown to be acetylated, the majority of which are involved in energy metabolism (Anderson and Hirschey 2012). Moreover, greater than 50% of these proteins are involved in glucose, fatty acid, and amino acid oxidation as well as the TCA cycle and oxidative phosphorylation (Anderson and Hirschey 2012), highlighting the potential importance of mitochondrial acetylation status on fuel metabolism. In aggregate, these data implicate acetyl-CoA as a critical player in metabolism.

1.2 Carnitine Trafficking of Acetyl Groups

Because acetyl-CoA and other acyl-CoA precursors cannot cross mitochondrial membranes, conversion of CoA metabolites to their respective carnitine esters is important for cellular and inter-tissue carbon trafficking. Muscle acylcarnitines are produced by several acyltransferase enzymes responsible for the interconversion of acyl-CoAs and acylcarnitine metabolites. The carnitine palmitoyltransferase (CPT) enzymes have specificity for long chain acyl-CoAs, while carnitine octanoyltransferase (CrOT) is principally a medium-chain acyltransferase. Alternatively, carnitine acetyltransferase (CrAT) has been shown to act primarily on short-chain acyl-CoAs. Acyltransferase enzymes differ from

one another primarily in the acyl-CoA binding pocket, with minor amino acid alterations dramatically changing substrate chain length specificity (Cordente et al. 2006, Cordente et al. 2004).

As a principal substrate of the acyltransferase enzymes, carnitine is best known for its role in shuttling long chain acyl-CoAs into the mitochondria for β -oxidation. Oxidation of acyl-CoAs is initiated by CPT1, which catalyzes the production of acylcarnitine and yields free CoA. Carnitine acylcarnitine translocase (CACT) then transports the acylcarnitine across the mitochondrial membrane, where carnitine palmitoyltransferase 2 (CPT2) converts it back into acyl-CoA utilizing an intramitochondrial pool of free CoA. Long chain acyl-CoAs are then further metabolized through mitochondrial β -oxidation (figure 1).

Notably however, in addition to its role in lipid oxidation, carnitine plays another comparatively understudied role in mitochondrial efflux of excess carbon fuels. The importance of this mechanism has been demonstrated in recent studies linking carnitine availability and glucose metabolism. These studies observed an improvement in whole body glucose tolerance in obese and diabetic rats after carnitine supplementation, an intervention that also caused a robust elevation of acetylcarnitine in both the plasma and urine (Noland et al. 2009). Because these studies and others have shown that perturbations in the carnitine pool may contribute to glucose intolerance (Noland et al. 2009, Powers et al. 2007), understanding the intricacies of carnitine homeostasis becomes important

in the understanding of mitochondrial metabolism. Carnitine availability is regulated at multiple levels, such as intestinal absorption, renal reabsorption, dietary intake, and endogenous biosynthesis (Steiber et al. 2004). Carnitine content is highest in foods from animal sources, such as red meat. Approximately 75% of total body carnitine comes from the diet in omnivorous humans, while vegetarians meet the majority of their carnitine needs via *de novo* biosynthesis (Steiber et al. 2004). Evidence suggests that carnitine synthesis can be compromised by depletion of essential co-factors in carnitine biosynthesis, such as iron, vitamin C, and α -ketoglutarate (Citak et al. 2006, Okamoto et al. 2006, Otsuka et al. 1999). Recent studies have additionally determined a regulatory role for PPAR α , a fatty acid-sensing nuclear receptor and regulator of lipid metabolism, in both carnitine synthesis and transport (van Vlies et al. 2007).

Once carnitine becomes available, it is transported into circulation via the organic cation family of transporters (OCT), which regulates both transport into and uptake from the circulation. OCTN2 is the primary carnitine transporter, located in the heart, skeletal muscle, kidney, placenta, small intestine and brain. Sodium-dependent transport of carnitine is achieved through OCTN2 with an apparent K_m of 4.3 μ M (Tamai et al. 1998). Interestingly, OCTN2 is also highly active in the transport of acetylcarnitine, with a K_m of 8.5 μ M (Ohashi et al. 1999). In aggregate, the carnitine pool is regulated at multiple levels including

intake, transport and synthesis. Recent evidence linking carnitine homeostasis to obesity and diabetes will be examined in future sections.

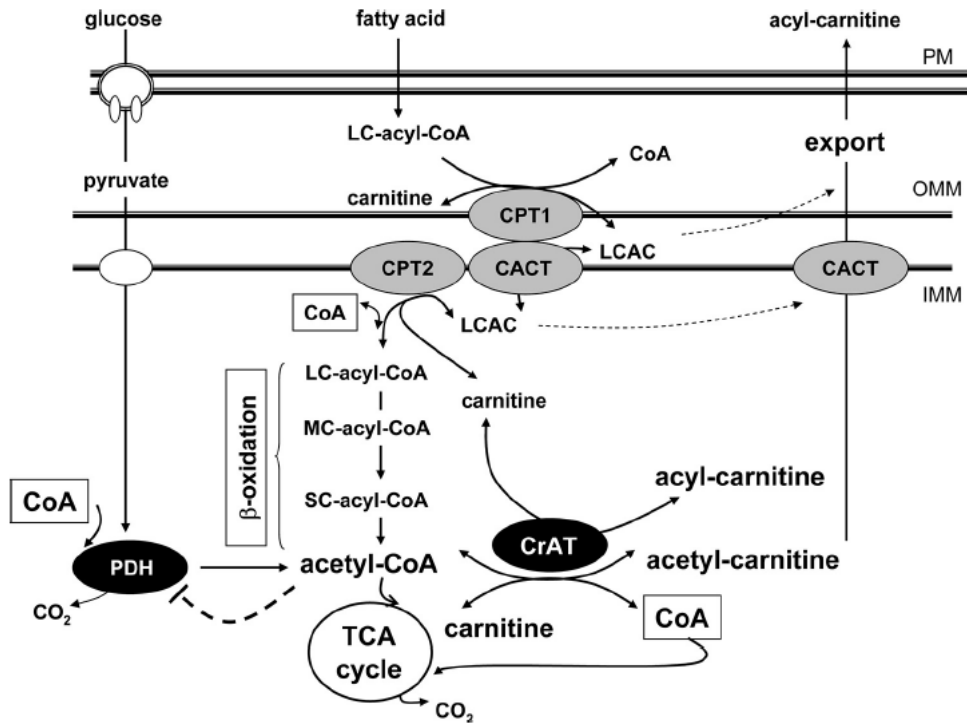


Figure 1: The Role of Carnitine and CrAT in Regulating Mitochondrial Energetics.

Long chain acyl-CoA must first be converted to its carnitine counterpart before transverse the mitochondrial membrane via CPT1 and CACT. CPT2 then regenerates the acyl-CoA within the mitochondria for entry into β -oxidation. Each successive cycle in β -oxidation produces a two carbon acetyl-CoA. Acetyl-CoA, also produced via glycolysis through PDH, can then enter the TCA cycle for further catabolism. CrAT, a matrix protein, converts acetyl-CoA into acetylcarnitine, which can efflux from the mitochondria, thereby relieving PDH inhibition should acetyl-CoA production exceed consumption.

1.3 Carnitine Acetyltransferase

Due to its ability to channel acetyl-CoA between the mitochondrial and cytosolic compartments, CrAT has long been thought to act in a buffering

capacity, freeing CoA for continued oxidative reactions through the generation of acylcarnitines which exit the mitochondria, efflux from the cellular compartments and enter the circulation (Brass et al. 1980, Carter et al. 1981, Brookelman et al. 1978). Because acetyl-CoA makes up greater than 90% of the total acyl-CoA pool, and because recent studies demonstrated the importance of acetylcarnitine efflux in obesity and diabetes, we examined the literature describing the CrAT enzyme.

Though the physiological role of CrAT remains uncertain, much is known about the biochemistry and enzymology of the enzyme. CrAT is present in both the mitochondrial matrix and peroxisomes. Peroxisomal CrAT is thought to be essential in allowing efflux of chain-shortened intermediates derived from very long-chain fatty acid catabolism from the peroxisomes to the mitochondria for further oxidation (Vanhove et al. 1991). Peroxisomal and mitochondrial CrAT are both transcribed by a single gene mapped to chromosome 9q34.1 (Corti et al. 1994, Corti et al. 1994b). It was found that the mitochondrial form of CrAT contains both peroxisomal and mitochondrial targeting sequences within the C- and N- terminal regions and that cleavage of the mitochondrial targeting sequence was required for peroxisomal targeting (Corti et al. 1994). This suggests that when present, the mitochondrial targeting sequence overrides peroxisomal targeting.

CrAT has been shown to be most abundant in skeletal muscle and heart, where it is primarily active in the mitochondria. CrAT has also been shown to be present in kidney and brain with low levels of enzyme in liver (Noland et al. 2009). Acetyl-CoA is largely consumed by the TCA cycle in muscle, while liver primarily utilizes acetyl-CoA for ketones and lipid synthesis. Therefore, though present in liver peroxisomes, CrAT is largely absent from liver mitochondria, likely because the presence of mitochondrial CrAT would disrupt ketone and lipid synthesis. Though little is known about the role of neuronal CrAT, in the two cases of human CrAT deficiency reported, both patients died in infancy with severe muscle derangements and neuronal defects (Melegh et al. 1999, DiDonato et al. 1979), suggesting a critical role for CrAT in muscle and brain metabolism.

Structural and functional data indicate that CrAT operates with a random bi-bi mechanism, meaning that the binding of two substrates yields two distinct products with random binding order (Colucci and Gandour 1987). Previous studies concluded that short-chain acyl-CoAs are preferred substrates for the CrAT reaction. Substrate specificity has been determined in purified heart CrAT (Huckle and Tamblyn 1983, Fritz et al. 1963), purified bovine sperm CrAT (Huckle and Tamblyn 1983), purified pigeon CrAT (Chase et al. 1967, Chase and Tubbs 1966), and more recently in rat CrAT overexpressed in yeast cells (Cordente et al. 2004). Though propionyl-CoA (three carbon acyl-CoA) and in

some models butyryl-CoA (four carbon acyl-CoA) are better substrates for the CrAT enzyme, acetyl-CoA is by far the most abundant acyl-CoA. Therefore, CrAT is best known for its role in regulating acetyl-CoA levels within the mitochondria.

Though substrate specificity has been well established, CrAT enzyme regulation is poorly understood. CrAT catalyzes the reversible reaction between short chain acyl-CoA and acylcarnitine with an equilibrium constant of 1.5–1.8 (Farrell et al. 1984, Pieklik and Guynn 1975). Therefore, enzyme activity has long been thought to be regulated by substrate/product concentrations within the mitochondrial matrix. Early studies demonstrated CrAT activity can be antagonized by long chain acyl-CoAs, though the relevance of this inhibition remains unclear and will be discussed in Chapter 3 (Huckle et al. 1983, Mittal et al. 1980, Chase et al. 1967). Both Mittal et al. (1980) and Chase et al. (1967) concluded that palmitoyl-CoA interacts with a hydrophobic region on CrAT that hinders carnitine binding in a reversible manner. Chapter 3 will discuss the possible acylation of the CrAT enzyme and its implications for mitochondrial fuel utilization. In aggregate, though much is known about the enzymology and biochemistry of the CrAT enzyme, its physiologic role in remains unknown. Therefore, we examine its role in obesity and diabetes in the following section.

1.4 Acetyl Group Imbalance in Insulin Resistance and Diabetes

Consumption of highly processed carbohydrate and fat-rich convenience food coupled with decreased physical activity has made obesity a global health concern. In the US alone, well over one third of the adult population is considered obese (Flegal 2012). Obesity is a primary physiologic component in what is known as the “metabolic syndrome,” a set of risk factors highly correlated with type 2 diabetes (Chan et al. 1994), and cardiovascular disease (Shan et al. 2009).

Insulin resistance, a hallmark of type 2 diabetes, is the failure of metabolic tissue to appropriately respond to the hormone insulin. The insulin-mediated capacity to clear blood glucose via skeletal muscle uptake and to limit glucose production by the liver is diminished, leading to compensatory insulin secretion and pancreatic β -cell expansion. This adaptation, in part, explains the abundance of individuals who are obese but not yet diabetic (Chan et al. 1994). The onset of type 2 diabetes results from the eventual impairment of β -cell function, resulting in an inability to produce insulin and maintain euglycemia (Pfeifer et al. 1981). Therefore, research targeting insulin resistance in the pre-diabetic state has become increasingly important.

One characteristic of the insulin resistant state is the inability of skeletal muscle to properly adjust to nutritional cues. This “metabolic inflexibility” refers to the apparent failure of insulin resistant animals to appropriately switch between

oxidation of fatty acid and glucose substrates (Kelley et al. 1999). In the fed state, healthy individuals experience a rise in glucose oxidation, whereas in the fasted state, lipids become the primary fuel. This concept, coined the “glucose-fatty acid cycle,” was first postulated by Randle et al. in 1963 and proposes that products of lipid oxidation such as acetyl-CoA, NADH and ATP suppress glucose metabolism via allosteric inhibition of PDH. More recent work demonstrated that the same set of allosteric inhibitors activate a family of PDH kinases, which phosphorylate and inhibit PDH (Sugden and Holness 2003). Randle additionally proposed a lipid product-mediated inhibition of phosphofructokinase-1 and hexokinase in the cytosol.

However, research interest has since shifted away from the Randle hypothesis. Recent studies have shown that decreased skeletal muscle glucose, the substrate of hexokinase, persists in diabetic subjects, suggesting that inhibition of hexokinase and phosphofructokinase does not drive insulin resistance (Cline et al. 1994). Therefore, research targeting insulin signaling and glucose uptake has gained momentum. Glucose uptake is driven by expansion of cell membrane GLUT4, which is set in motion by insulin signaling. This cascade is initiated when insulin binding results in auto-activation of the insulin receptor followed by tyrosine phosphorylation of insulin receptor substrate 1 (IRS-1), leading to translocation of GLUT4-containing vesicles to the sarcolemma. Persistent serine phosphorylation by serine kinases, such as protein kinase C

(PKC) and c-jun N-terminal kinase (JNK), negatively regulate both the insulin receptor and IRS-1 in the insulin resistant state (Morino et al. 2006). Bioactive lipid signaling molecules have been implicated in this role. Diacylglycerols, long chain acyl-CoAs and ceramides activate these serine kinases along with a series of proinflammatory signals (Kim et al. 2007, Senn 2006, Shi et al. 2006), resulting in decreased insulin signal transmission and glucose uptake (Hirosumi et al. 2002 and Shulman et al. 2000). In aggregate, these studies and others led to a model in which muscle mitochondria diverts fatty acids away from oxidation and toward the formation of toxic lipid metabolites which antagonize insulin signaling.

1.5 Metabolic Profiling Provides New Insights into the Randle Cycle

Several lines of evidence argue against ectopic fat accumulation as a primary cause of insulin resistance. For example, an accumulation of intramuscular fatty acids is present in insulin sensitive athletes (Goodpaster and Kelley 2002). Moreover, exercise intervention improved insulin sensitivity in type 2 diabetic patients without a subsequent reduction in long chain acyl-CoA or diacylglycerol levels (Bruce et al. 2004) thereby calling into question the idea that mitochondrial dysfunction is a core cause of fat accumulation and muscle insulin resistance.

Recently studies have refocused attention on the link between mitochondrial energetics and insulin signaling with the observation that lipid-

induced derangements in substrate switching occurred within isolated mitochondria (Noland et al. 2009, Koves et al. 2008). In support of this model, use of targeted mass spectrometry-based metabolic profiling suggested that excessive β -oxidation might be a root cause of insulin resistance. These studies demonstrated that insulin resistant skeletal muscle was marked by excessive entry into β -oxidation leading to elevated incomplete oxidation of lipid fuels and coincident lowering of tricarboxylic acid cycle (TCA cycle) intermediates (Koves et al. 2008). Importantly, incomplete oxidation, or the accumulation of medium and short chain acylcarnitines, has become recognized as a signature of obese and diabetic muscle (Boyle et al. 2011, Kovalik et al. 2011, Thyfault et al. 2010, Noland et al. 2009, Koves et al. 2008).

These data linking excessive carbon load to insulin resistance are in line with emerging evidence that muscle glucose intolerance is driven by mitochondrial stress. Indeed, recent studies suggest that an excess in carbon load in the absence of ATP demand leads to persistent pressure on the electron transport chain, yielding reactive oxygen species (ROS) production and disrupted redox balance (Bloch-Damti and Bashan 2005, Evans et al. 2005). ROS generation is thought to primarily occur within the electron transport chain (ETC) and has been demonstrated to principally result from excessive fatty acid-supported respiration (Anderson et al. 2009). Mitochondrial ATP generation relies on the ETC using both an electron donor coming from fuel, and oxygen, the

electron acceptor. Electron donors in the form of the reducing equivalents NADH and FADH₂ drive the electron transport chain. Both β -oxidation and the TCA cycle produce FADH₂ which donates electrons to the electron-transfer flavoprotein (ETF) and into to the ETC via the ubiquinone (Q) cycle. Likewise, NADH, produced by β -oxidation, PDH and multiple enzymes in the TCA cycle, enters the ETC at complex I and provides electrons into the Q cycle. ROS production is high when ATP demand is exceeded by electron flux into the ETC. This causes heightened backpressure on complex I, resulting in increased electron leak and ROS production (reviewed in Fisher-Wellman and Neuffer 2012, Muoio and Neuffer 2012). ROS generation has been shown to activate multiple serine kinases and transcription factors implicated in aberrant insulin signaling (Bloch-Damti and Bashan 2005, Chakraborti and Chakraborti 1998), thereby highlighting the association between ROS production and glucose intolerance.

Further linking acetyl group accumulation and insulin signaling is the finding that overnutrition and insulin resistance lead to changes in the mitochondrial acetylation state (Hirschey et al. 2009, Huang et al. 2010). An estimated 35% of mitochondrial proteins have been shown to be acetylated, with acetylated proteins present in every major pathway in mitochondrial metabolism (Anderson and Hirschey 2012). Three mitochondrial sirtuin deacetylase enzymes have been identified to be important for regulation of the mitochondrial acetylation state. These will be discussed in more detail in the future directions

section. The primary deacetylase, SIRT3, has been shown to act on long chain acyl-CoA dehydrogenase (LCAD) and superoxide dismutase (SOD), deacetylating and increasing enzyme activity (Hirschey et al. 2010, Qui et al. 2010). Interestingly, chronic nutrient overload led to decreased SIRT3 protein and mRNA in both liver (Hirschey et al. 2011, Bao et al. 2010) and skeletal muscle (Jing et al. 2011), resulting in mitochondrial hyperacetylation. These data suggest a link between the mitochondrial acetylation and glucose intolerance. In support of this hypothesis, SIRT3 knockout mice developed insulin resistance with aging (Hirschey et al. 2010, Jing et al. 2011, Qui et al. 2010). These data support the idea that nutrient-induced acetyl-CoA accumulation might play a causal role in the metabolic syndrome.

In aggregate, caloric excess results in the production of potentially harmful intermediates which inhibit PDH, increase the mitochondrial acetylation state and overwhelm the ETC, producing ROS. These studies support the idea that mitochondrial stress brought on by excess carbon load results in derangements in glucose metabolism. Therefore, efflux of these metabolite intermediates out of the mitochondria could provide a “safety valve”, critical in the prevention of nutrient-induced mitochondrial stress.

1.6 Studies in CrAT Knockout Mice Support the Mitochondrial Stress Theory

Because previous studies suggested that CrAT-mediated acetyl-CoA regulation might play a role in defending whole body glucose homeostasis (Noland et al. 2009), we used recombinant adenovirus manipulations of the CrAT protein in primary human skeletal muscle myocytes to examine the effect of CrAT activity on fuel metabolism. CrAT knockdown resulted in elevated fatty acid oxidation, while glucose uptake was diminished (Figure 2a). Moreover, overexpression of CrAT resulted in decreased fatty acid oxidation (Muio et al. 2012), and increased glucose uptake (Noland et al. 2009; Figure 2b) in primary human skeletal muscle cells. These data highlight the importance of CrAT activity in glucose metabolism.

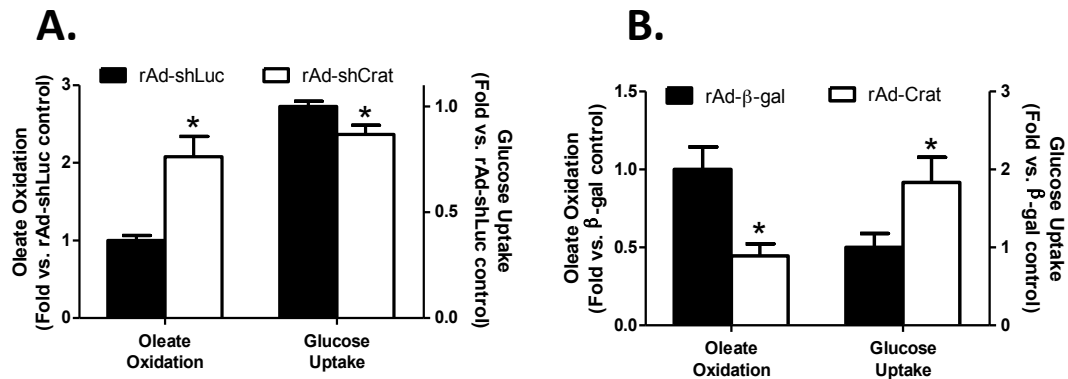


Figure 2: CrAT Counterregulates Glucose and Fatty Acid Metabolism in Primary Human Skeletal Myocytes

Treatment of primary human skeletal myocytes with rAD-shCrAT increased oxidation of 100 μM [$1\text{-}^{14}\text{C}$]oleate to CO_2 and decreased uptake of [3H]-2-deoxy-glucose (A), while overexpression of CrAT using rAD-CrAT decreased oxidation of 100 μM [$1\text{-}^{14}\text{C}$]oleate to CO_2 and increased uptake of [3H]-2-deoxy-glucose (B).

In order to assess the role of CrAT in whole body fuel metabolism, we generated a muscle specific CrAT knock out mouse model. CrAT deficiency resulted in reduced muscle acetylcarnitine content, suggestive of an accumulation in mitochondrial acetyl-CoA. These derangements in CrAT-mediated acetylcarnitine efflux were coupled with an insulin resistance phenotype and an inability to appropriately transition from fatty acid to pyruvate oxidation (Figure 3).

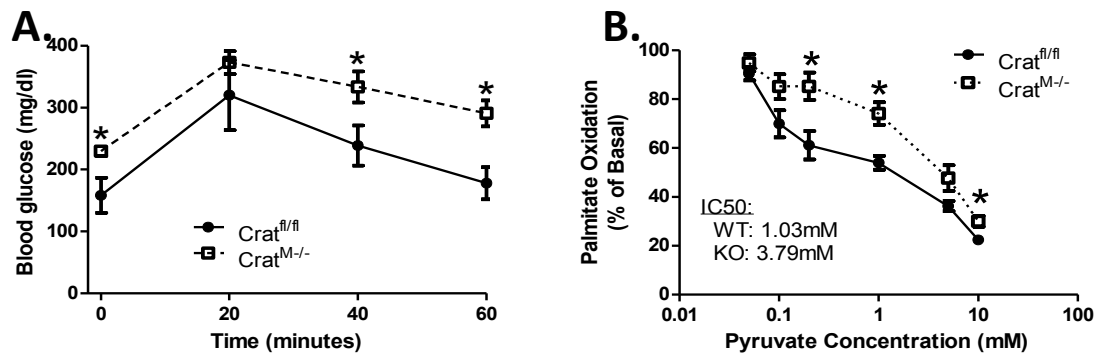


Figure 3: CrAT Deficiency Affects Whole Body and Mitochondrial Homeostasis

CrAT muscle specific knockout mice (CrAT^{M-/M-}) and control littermates (CrAT^{fl/fl}) were used to perform glucose tolerance tests after a low fat control diet (A). Isolated gastrocnemius muscle mitochondria were used to assess the dose-dependent pyruvate inhibition of fatty acid oxidation using 300 μ M [1-¹⁴C]palmitate. Results were normalized to basal oxidation rates and IC50 values were calculated for the inhibitory effect of pyruvate on palmitate oxidation.

Because acetyl-CoA is a potent inhibitor of PDH activity, we considered the idea that CrAT-mediated acetyl-CoA regulation could become imperative for PDH activity and glucose oxidation. As discussed previously, studies from Noland et al. 2009 supported this model, demonstrating that genetic and nutritional manipulations that enhance or diminish forward flux through the CrAT reaction resulted in corresponding changes in PDH activity and glucose disposal. In further support of this hypothesis, deletion of CrAT resulted in complete loss of carnitine-stimulated PDH activity in skeletal muscle mitochondria (Figure 4). These data suggest that CrAT-mediated acetyl-CoA regulation may be modulating PDH activity to increase glucose metabolism.

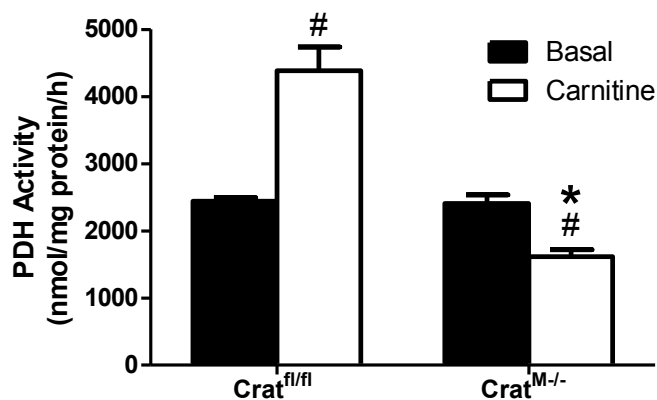


Figure 4: CrAT Regulates PDH Activity

PDH activity was determined by measuring $^{14}\text{CO}_2$ produced from 1 mM $[1-^{14}\text{C}]$ pyruvate \pm 5 mM carnitine in isolated gastrocnemius muscle mitochondria from CrAT^{M-/-} and CrAT^{fl/fl} littermates.

To further implicate a role for CrAT in combating nutrient stress, age matched human subjects with modestly elevated blood glucose underwent six months of carnitine supplementation (Noland et al. 2009). Importantly, carnitine permitted efflux of excess mitochondrial acetyl-CoA and enhanced insulin action. PDH activity and circulating acetylcarnitine were elevated while blood glucose and insulin were normalized. In aggregate, these data suggest that CrAT-mediated acetyl-CoA regulation combats nutrient-induced mitochondrial stress and may serve as a therapeutically relevant target of metabolic inflexibility.

1.7 Acetyl-CoA Buffering in Exercise

In addition to nutritional stress, exercise represents another physiological condition that causes dramatic swings in acetyl-CoA production and consumption. Rapid adjustments in the rate of mitochondrial ATP production depend on availability of oxygen as the final electron acceptor and a steady supply of electron donors. Acetyl-CoA is the primary substrate of the TCA cycle which supplies reducing equivalents as electron donors to the electron transport chain. Therefore, a deficit in acetyl-CoA supply would be predicted to limit the rate of oxidative ATP production.

Two classifications of ATP production exist in exercise, aerobic or oxygen-dependent and anaerobic or oxygen independent ATP production. During exercise, the majority of ATP is delivered to myosin ATPases, which consume ATP to facilitate contraction via power strokes involving actin and myosin. However, at rest, enough ATP exists to facilitate only a few seconds of contraction if not replenished (Hargreaves and Spriet 2006). Therefore, skeletal muscle production of ATP must match energy demand. While anaerobic ATP production provides an elevated rate of ATP compared to aerobic production, (up to 6 times faster), it is not sustainable. Additionally, accumulation of the lactate by-product of anaerobic glycolysis decreases muscle pH, thereby inhibiting enzymes in glycolysis and contributing to muscle cramping (Hargreaves and Spriet 2006). Therefore, aerobic ATP generation is the preferred form of energy

production for most exercise states. However, aerobic ATP generation cannot account for rapid fluctuations in ATP demand.

Anaerobic ATP production predominantly occurs in type II muscle fibers which can be further subdivided into type IIA, or fast oxidative and type IIB or fast glycolytic fibers. The majority of anaerobic ATP generation comes from glycolysis, a cytosolic pathway ending in lactate production. Regulation of glycolysis is achieved primarily through phosphofructokinase activity, the kinase responsible for catalyzing the major regulated step in glycolysis. Accumulation of ADP and AMP activate this enzyme, stimulating glycolysis, while ATP is inhibitory. Provision of glucose for glycolysis is achieved through potent stimulation of glucose uptake into the muscle as well as elevations in glycogen utilization occurring during exercise. If blood glucose concentrations are maintained during contraction, glucose uptake continues to increase (Angus et al. 2002). However, if blood glucose is not maintained, glucose uptake peaks and begins to decline as levels become limiting (Katz et al. 1991, Ahlborg and Felig, 1982, Ahlborg et al. 1974). Therefore, glycogen stores become essential for maintenance of glycolysis. Glycogen utilization, also stimulated by contraction, is at an energetic advantage, yielding three ATP per glucosyl group compared to the two ATP per glucose molecule. The importance of both blood glucose and glycogen in supplying ATP is emphasized by observations that fatigue associated with prolonged strenuous exercise is associated with glycogen

depletion and/or hypoglycemia (Coggan and Coyle 1987, Coyle et al. 1986, Coyle et al. 1983, Hermansen et al. 1967, McConell et al. 1999). However, anaerobic ATP can be generated from non-glycolysis sources.

In extreme exercise, a small amount of ATP generation can come from the adenylate kinase reaction, which transfers a high energy phosphate from ADP to produce ATP and AMP. AMP is then degraded to IMP in order to keep the adenylate kinase reaction favoring the production of ATP. ATP can additionally be derived from the phosphocreatine (PCr) reaction wherein the conversion of PCr and ADP to creatine and ATP is catalyzed by creatine kinase. Though resting stores of PCr can serve as a rapid source of ATP generation, contraction cannot be sustained for longer than a few seconds using this fuel source alone (Hargreaves and Spriet 2006).

In addition to its role in buffering changes in ATP demand, the creatine kinase reaction is critical for the rapid and efficient trafficking of ATP from its production site in the mitochondria to ATPase in the myofibrils during work. It has been estimated that as much as 80% of ATP transfer from the mitochondria to cytoplasm occurs through creatine kinase-dependent cycling (Aliev et al. 2011, Seppet et al. 2001, Guzun et al. 2012). Mitochondrial, cytosolic and myofibril creatine kinase isoforms couple ATP trafficking to contractile machinery. In support of this model, sensitivity to ADP in skeletal muscle fiber bundles was

greatest when both contractile signals and maximal creatine kinase activity were present (Perry et al. 2012).

Anaerobic ATP production becomes vital during rapid changes in ATP demand and at the onset of exercise. A lag in oxygen-dependent provision of energy has been observed during initiation of exercise. This phenomenon, called the oxygen deficit, has been described using measurements in VO_2 kinetics during contraction to reveal a disconnect between oxygen uptake and ATP demand during the transition from low to high workloads (Figure 5; adapted from Hargreaves and Spriet 2006). Two theories have been proposed to account for this lag in oxygen-dependent ATP generation. The first suggests a limitation in oxygen delivery to ETC, while the second proposes a deficit in acetyl group availability, also referred to as metabolic inertia. Acetyl-CoA, the common metabolic intermediate of glucose, amino acid and fatty acid catabolism, fuels TCA cycle production of reducing equivalents. Because these reducing equivalents drive oxidative phosphorylation, a deficit in acetyl group production has been suggested to limit ATP generation. In support of this hypothesis, recent work demonstrated that expansion of the acetyl-CoA pool improved exercise performance (Howlett 1999, Timmons 1998, Timmons 1996), linking acetyl-CoA buffering to contractile capacity. In chapter 4 we examine the metabolic inertia theory by testing the role of CrAT-mediated skeletal muscle uptake and/or production of acetyl groups during exercise.

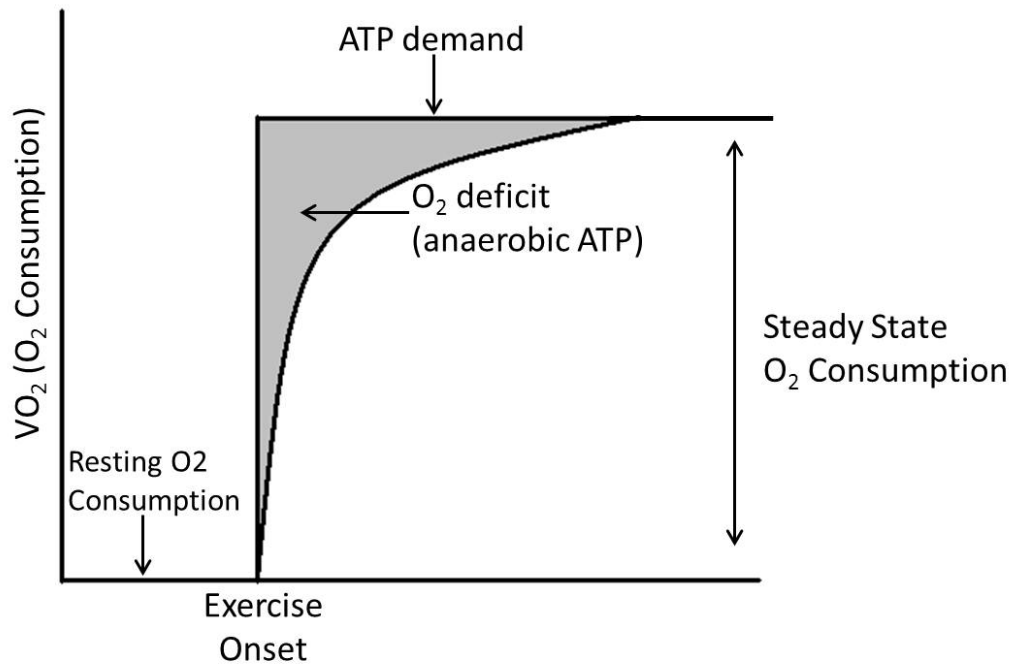


Figure 5: The Oxygen Deficit

Diagram representing the lag in oxygen-dependent ATP production at the onset of exercise. Anaerobic ATP production is necessary to meet the energy requirements during this period.

1.8 Project Goals

The studies presented herein were designed to address the physiologic significance of CrAT-mediated acetyl-CoA buffering. These data demonstrate the critical role of CrAT in fine tuning the acetyl-CoA balance and suggest that absence of the CrAT enzyme results in glucose intolerance and fatigue. Chapter 3 discusses the involvement of CrAT-mediated acetyl-CoA regulation in glucose homeostasis. Alternatively, work discussed in Chapter 4 highlights the essential role of acetyl-CoA buffering in exercise capacity and fatigue. These data address

the significance of acetyl-CoA regulation in multiple scenarios and suggest its therapeutic potential.

Because acetyl-CoA regulation has been shown to play a role in glucose metabolism and metabolic flexibility (Noland et al. 2009), and because CrAT regulates this metabolite pool, understanding the regulation of this enzyme may be critical to our understanding of metabolic disease. Though studies focused on discerning CrAT enzyme structure and activity have been reported since the 1960s, little is known about its regulation. Because CrAT is a freely reversible enzyme, its activity has long been thought to be regulated by substrate/product concentrations within the mitochondrial matrix. Chapter 3 suggests that while CrAT-mediated acetyl-CoA regulation facilitates the fasting to fed transition (Muoio et al. 2012), fatty acids counter-regulate the enzyme, allowing fat to be the primary fuel source. We suggest that lipids regulate CrAT within cultured cells and animal models both by sequestering carnitine into LCACs and by direct non-competitive binding of long chain acyl-CoAs, thereby resulting in the accumulation of acetyl-CoA which causes PDH inhibition and mitochondrial stress. Results from these studies indicate that lipid-induced antagonism of acetyl-CoA efflux may contribute to low PDH activity and glucose disposal in the context of obesity and diabetes.

Alternatively, we investigated the importance of acetyl-CoA buffering during exercise. Though multiple studies have focused on the forward CrAT

reaction (conversion of acetyl-CoA to acetylcarnitine), very little is known about the significance of the reverse reaction, despite the fact that CrAT is a freely reversible enzyme. Additionally, though CrAT is highly expressed in the skeletal muscle and heart, tissues specifically designed for energy output, little is known about the role of CrAT during exercise and other circumstances of energy demand. Therefore, we developed a skeletal muscle-specific CrAT knock out mouse model to analyze the role of CrAT in acetyl-CoA buffering during exercise. Chapter 4 suggests that CrAT-mediated maintenance of the acetyl-CoA pool may be critical to combat exercise-induced fatigue.

In summary, we suggest the seminal importance of CrAT in fine-tuning the acetyl-CoA pool. These data demonstrate that CrAT deficiency leads to glucose intolerance and fatigue. Herein we discuss the important clinical implications of targeting the CrAT reaction and discuss potential future areas of study.

2 Methods

2.1 Generation of *CrAT*^{SM-/-} Mice:

As described by Muoio et al. 2012, *CrAT*^{fl/fl} mice were created using a conditional targeting vector was constructed using recombineering system. Isogenic DNA containing *crat* exons 3-13 was retrieved from genomic colony RP24-249K14 of C57BL/6J BAC genomic library via gap repair. The first loxP site was inserted into intron 8 and the loxP-neo-loxP into intron 11 via homologous recombination in E.Coli so that the exons 9-11 (1.0kb) were flanked by the first two loxP sites to generate *crat* targeting construct. Key elements were then confirmed by sequencing. This strategy deleted exons 9-11 and disrupted the coding sequence of exons 12-14 to obtain complete inactivation of CrAT enzyme function. For gene targeting, Not I-linearized *crat* targeting vector DNA consisting of 6.3kb 5'sequences (upstream of the first loxP site) and 1.8kb 3' sequences was electroporated into C57BL/6J derived Bruce-4 embryonic stem (ES) cells. Correct homologous recombination in targeted clones was identified with Fidelity PCR at the 5'-end and 3'-end. The fragments produced from Fidelity PCR with these primers were sequenced to further confirm the correctness of recombination event and the location and sequence of loxP sites. Targeted ES were injected into albino B6 blastocysts (B6(Cg)-Tyrc-2J/J Jax Stock Number: 000058). The heterozygotes (*Crat*^{fl/neo}+) were bred with ubiquitously expressed Cre (*Ella-Cre*) to generate heterozygous mice (*Crat*^{fl/+}) without

PGK-neo. To generate muscle specific knockout mice for CrAT, *Cratflox/+* were bred to myogenin-Cre recombinase mice, which were a generous gift from Eric Olson (UT Southwestern.) These mice were backcrossed for 10 generations to C57BL/6J mice before breeding to floxed CrAT. CrAT KO mice (*Crat flox/flox;Myo-Cre*) and control littermates (*Crat flox/flox*) were fed a standard chow diet (Purina Rodent Chow no. 5015, Purina Mills, St. Louis, MO, USA.)

2.2 Mitochondrial Isolation:

Skeletal muscle mitochondria were prepared based on the procedure of Kerner 2001 with some modifications. Mouse gastrocnemius muscles were removed under anesthesia and placed in ice cold KMEM buffer (100mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO₄, pH 7.4). Tissues were cleaned, blotted, weighed, finely minced, and suspended at a 10-fold dilution in KMEM plus 1mM ATP. The suspension was homogenized on ice using ten passes with a Potter-Elvehjem homogenizer. KMEM/ATP buffer supplemented with 0.2% BSA was then added for a total 20 fold total dilution. The homogenate was centrifuged at 500xg for 10 min at 4 °C. The supernatant was then centrifuged at 10,000xg for 10 minutes at 4 °C and the pellet was resuspended in 500µL of KMEM/ATP/BSA buffer and centrifuged at 7000xg for 10 min at 4 °C to wash. The pellet was then resuspended in 500µL KMEM and centrifuged at 7000xg for 10 min at 4 °C, the resulting pellet being the mitochondrial fraction. The final pellet was resuspended in CellLytic lysis buffer (Sigma Chemicals, St. Louis, MO) and processed by

freeze fracturing three times and sonication at five times one second pulses on setting five using a Sonic Dismembrator from Fisher Scientific.

2.3 Carnitine acetyltransferase activity:

Carnitine-dependent conversion of acetyl-CoA to acetylcarnitine and free CoA was measured as previously described with minor modifications (1). Acetyl-CoA and 0.1 mM DTNB [5,5'-dithiobis(2-nitrobenzoic acid)] were combined with purified enzyme, cell lysates or isolated mitochondria. The assay buffer included 50 mM Tris and 1 mM EDTA in water at pH 7.8. CrAT activity was determined spectrophotometrically at 412 nm by evaluating the rate of reduction of DTNB by free CoA on a Spectramax M5 spectrophotometer (Molecular Devices). Samples were read for 2 minutes in the absence of carnitine to determine a baseline rate. Reactions were started with the addition of 5 mM L-carnitine (unless stated otherwise) and monitored every 20 seconds for 10 minutes at 25°C. The 2 minute baseline rate was then subtracted from the final linear rate to yield a corrected rate. Activity calculations were made using an instrument specific extinction coefficient for TNB of $16,029 \text{ M}^{-1}\text{cm}^{-1}$ determined using L-glutathione as a CoA donor and correcting for a path length of 0.641 cm (for a 0.2 ml reaction volume in NUNC 96-well plates). All substrates were purchased from Sigma and re-constituted in activity buffer.

2.4 Cell Culture:

Primary human skeletal myocytes were grown and differentiated as previously described (Muio et al.2002) in medium supplemented with 100 μ M L-carnitine (Sigma; ST. Louis, MO). Human samples were obtained from the vastus lateralis muscle via needle biopsy at weights between 100 and 200 mg. Samples were rapidly placed in ice-cold Dulbecco's modified Eagles media (DMEM; Life Technologies; Gaithersburg, MD), and trimmed of connective tissue and fat . Trypsin digestion was used to isolate satellite cells and plated for 1-3 hours in 3.0 mL growth media (DMEM with human skeletal muscle SingleQuotes from BioWhittaker (Walkersville, MD). Growth media was supplemented with 10% fetal bovine serum (FBS), 0.5 mg/ml BSA, 0.5 mg/ml fetuin, 20 ng/ml human epidermal growth factor, 0.39 g/ml dexamethasone, and 50 g/ml gentamicin/amphotericin B on uncoated T-25 tissue culture flasks to remove fibroblasts. Cells were then transferred to a type 1 collagen-coated T25 flask and cultured at 37°C in a humidified incubator with 5% CO₂. After the cells reached about 70% confluence, myoblasts were cultured on three collagen-coated p-100 plates. When cells reached 70% confluence (generally 5-10 million cells), 0.5 million cells per cryovile in were frozen down in 95% growth media, 5% DMSO overnight at -80, then in liquid nitrogen. For experimental purposes, each cryovile was thawed in a T-75 uncoated flask in growth media and split onto collagen-coated plates. Once at 80% confluence, differentiation was induced by changing

to low-serum media containing 2% heat-inactivated horse-serum (Hyclone (Logan, UT), 0.5 mg/ml BSA (essentially fatty acid free; Sigma; St. Louis, Mo), 0.5 mg/ml fetuin (Sigma; St. Louis, Mo), and 50 g/ml gentamicin/amphotericin B (Life Technologies Gaithersburg, MD). Media was changed every 2–3 days. Cytomegalovirus (CMV) promoter-driven recombinant adenoviruses encoding either β -galactosidase (rAdCMV- β -gal) or Myc-tagged rat carnitine palmitoyltransferase b (rAdCMV-CPT-1) were constructed, amplified and purified according to (Becker et al.1994). On differentiation day 3, myotubes were exposed to 5.3×10^{-3} infectious units/cm² rAdCMV-CPT-1. Metabolic analyses were performed 7d after virus treatment.

2.5 DCFA Confocal Imaging:

Primary human skeletal muscle or L6 skeletal muscle cells were used for confocal imaging. Upon 80% myoblast confluence, 100 μ M L-carnitine or water vehicle was added to differentiation media described in the *cell culture* section above. Media was changed every other day. On differentiation day 5, 250 μ M buthionine sulfoximine (BSO) was added as a 24 hour treatment to decrease total glutathione levels in the cell. On differentiation day 6, cells were washed twice with PBS and then loaded with 5 μ M DCFA in differentiation media without phenol red for 30 minutes at 37°C. A 5mM stock solution of DCFDA (Sigma; St. Louis, Mo) was made fresh each day in ethanol. Each treatment (+/- carnitine, +/- BSO), was present during the 30 minute DCFA treatment. Once DCFA was

added, cells were kept in the dark. After the 30 minute DCFA treatment, cells were washed twice with PBS and placed in phenol red free differentiation media +/- carnitine and +/- BSO to keep treatment conditions consistent. Cells were imaged in a Leica SP5 confocal microscope in a Ludin cube live cell chamber (humidified environment containing 5% CO₂ at 37°C). An argon 488 nm laser was used for DCFA detection. Laser exposure and smart gain fields were kept consistent between images.

2.6 Western blots:

Protein was isolated using CellLytic lysis buffer (Sigma Chemicals, St. Louis, MO). A bicinchoninic acid (BCA) kit (Sigma Chemicals, St. Louis, MO) was used to quantify protein. Protein (50µg from cell/tissue lysates) was separated by SDS-PAGE, transferred to nitrocellulose, and incubated with antibodies prepared with 5% milk in TBS-Tween. Secondary antibodies were HRP-conjugated and ECL detection reagent (Pierce, Rockford, IL) was used to visualize protein bands. The primary CrAT antibody was acquired as a generous gift generated in the laboratory of Dr. Fausto Hegardt. MemCode was used to determine protein loading and was obtained from Thermo scientific.

2.7 RNA Analysis:

RNA was isolated from mixed gastrocnemius using the total RNA isolation kit (Qiagen, Valencia, CA). RNA quality and quantity were determined using a

NanoDrop 8000 (Thermo Scientific). Using the IScript cDNA synthesis kit (Bio-Rad, Hercules, CA), cDNA was made using 1µg RNA in a 20µL reaction volume. cDNA was then diluted 5 fold for RT-PCR. mRNA was assessed via RT-PCR using a Prism 7000 and TaqMan pre-designed/pre-validated FAM-labeled Assays-on-Demand (Applied Biosystems, Foster City, CA), and real-time master mix. Data was normalized to VIC-labeled 18S endogenous control gene expression (Applied Biosystems) using a duplexed RT-PCR reaction.

2.8 Metabolite Analysis:

Tissue and plasma samples were processed and analysed by the metabolomics and proteomics core facility at the Stedman Center core facility. Acylcarnitine measurements were made using flow injection tandem mass spectrometry and sample preparation methods described in (An et al.2004 and Haqq et al.2005.) Data were acquired using the Micromass Quattro Micro TM systems and a model 2777 autosampler, a model 1525 HPLC solvent delivery system and a data system controlled by MassLynx 4.0 operating systems (Waters, Millford, MA). Acyl-CoA measurements were analyzed using a method based on a the protocol used in Magnes et al. which employs the extraction protocol described in Deutsch et al. CoAs were further purified by solid phase extraction described in Minkler et al. Acyl CoAs were analyzed by flow injection using positive electrospray ionization and a Xevo TQS, triple quadrupole mass spectrometer (Waters, Milford, MA) using methanol/water (80/20, v/v) containing 30 mM

ammonium hydroxide as the mobile phase. Spectra were acquired in the multichannel acquisition mode monitoring the neutral loss of 507 amu (phosphoadenosine diphosphate) and scanning from m/z 750- 1060. Heptadecanoyl CoA was used as an internal standard. Endogenous CoAs were quantified using calibrators prepared by spiking skeletal muscle homogenates with known CoAs (Sigma, St. Louis, MO) having saturated acyl chain lengths between C₀- C₁₈. Corrections for the heavy isotopes to the adjacent m+2 spectral peaks for each chain length cluster were made empirically by referring to the observed spectra for the analytical standards.

2.9 Phosphocreatine and Creatine Content:

Phosphocreatine (PCr) and creatine analysis was measured according to Bergmeyer 1974 and Harris et al.1974. Before analysis was conducted, a skeletal muscle perchloric acid (PCA) extraction was completed. 0.5M PCA (10M EDTA) was added at 60x sample volume to ground freeze-dried samples and vortexed. These samples were then stored on ice for 5 min with intermittent vortexing throughout. Samples were then centrifuged at 7000 rpm for 5 min (4°C). 80% of the PCA volume added was then removed and ¼ of this volume was added back with 2.2M KHCO₃. Samples were then vortexed 2-3 times on ice until the bubbling stopped. The samples were then centrifuged at 7000 rpm for 15 min (4°C).

PCr analysis was run at 340nm in cuvettes with 25 μ L muscle extract added to 225 μ L reagent. The reagent consisted of 100mM triethanolamine (pH 7.5,) 10mM mg(Ac)₂4H₂O, 1mM EDTA.Na₂.2H₂O, 1mM DTT, 1mM NADP.Na₂.4H₂O (this must be made fresh,) 0.04mM ADP.Na₂.2H₂O, 5mM glucose, 0.67 μ L per sample G6PDH (Roche, Basel Switzerland) and 178.75 μ L per sample water. Cuvettes were then read for background absorbance. 2 μ L hexokinase (Roche, Basel Switzerland) and 2 μ L CPK at 15mg/ml (Sigma; St. Louis, Mo) were then added. PCr was measured after a 4-5 minute reaction.

Creatine analysis was run at 340nm in cuvettes. 20 μ L muscle extract was added to 250 μ L reagent. The reagent consisted of 100mM glycine, 5mM mg(Ac)₂4H₂O pH 9, 30mM KCl, 1.5mM ATP.Na₂.3.5H₂O, 1mM PEP, 0.15mM NADH (this must be made fresh,) 0.45 μ L per reaction LDH (Roche, Basel Switzerland), 0.45 μ L per reaction pyruvate kinase (Roche, Basel Switzerland) and 134.5 μ L per reaction H₂O. Cuvettes were read for background absorbance. 10 μ L creatine phosphokinase (Sigma; St. Louis, Mo, made up at 15mg/mL in H₂O) was added and creatine was assessed at 340nm.

2.10 Glycogen Content:

Glycogen was assessed using approximately 20 mg of powdered skeletal muscle. The powdered tissue was added to 0.5 ml of 1 N HCl secured in 2.0-ml screw top tubes and homogenized at 25K RPM for 30 seconds using a sawtooth, rotor/sator homogenizer VDI-12 with 5 mm probe (VWR; Radnor, Pennsylvania).

The samples were then boiled for 90 minutes. The tubes were cooled to room temperature and 0.5 ml of 1N NaOH was added. Samples were then spun at 14,000xg for 10 minutes at room temperature. Glycogen content was assessed in the sample supernatant. 20 μ L in triplicate was assessed at 340nm after addition of glucose assay reagent (Sigma; St. Louis, Mo). The glucose standard curve was made up using powdered glucose (Sigma; St. Louis, Mo), in 1:1 acid: base (pH neutral) serially diluted from 5 mM to 0.195 mM glucose. Glycosyl units were calculated based on the 1 mL starting supernatant and expressed as μ mole glycosyl unit per gram starting wet weight of tissue.

2.11 Lactate Content:

Lactate was assessed at 340nm. Lactate assay buffer consisted of 175mM hydrazide sulfate, 68mM glycine, 2.9mM EDTA, and 11.3mM NAD⁺ (which must be added fresh each day,) pH 9.5. Lactate standard (Sigma; St. Louis, Mo) was made up at 3, 1.5, 0.75, 0.375 and 0.1875mM. 20 μ L sample or standard was added with 200 μ L assay buffer. Background absorbance was measured. 10 μ L LDH enzyme (Sigma; St. Louis, Mo,) made up at 500 units/ml in water, was then added rapidly and absorbance was monitored for 60 minutes. Upon kinetic plateau, final absorbance was taken. Lactate was determined according to the standard curve.

2.12 Nucleotide Profiling:

Nucleotides were measured by a method modified from a previously reported LC-MS/MS method (Cordell et al, 2008). An EDL (10-13 mg) was transferred to a 2 mL tube prechilled in liquid nitrogen. A solution of methanol (200 μ L) containing internal standards was added as well as a prechilled 5 mm glass bead (Glen Mills Inc., Clifton, NJ, USA). Tissues were lysed in a Qiagen Tissue Lyser II for 2 x 30s at a frequency of 30/s. Chilled water was added to bring the mixture to 300 μ L and the samples were allowed to extract for 15 min at -20 $^{\circ}$ C. Hexane (300 μ L) was added to each tube, and the tubes were thoroughly mixed. The tubes were then centrifuged and the bottom layer collected in a fresh tube and centrifuged again. The supernatant was transferred to a 96-well plate for analysis by liquid chromatography tandem mass spectrometry. Chromatographic separations were performed using an Agilent Technologies (Santa Clara, CA) 1200 HPLC system and a Chromolith FastGradient RP-18e 50-2mm column (EMD Millipore, Billerica, MA, USA) under the following conditions. Injection volume was 2 μ L. Mobile phase A was 95% water 5% methanol and 5 mM dimethylhexylamine. Mobile phase B was 20% water 80% methanol and 10 mM dimethylhexylamine. Flow rate was set to 0.3 mL/min and column temperature was 40 $^{\circ}$ C. A 22 min gradient method (t=0, %B=0; t=1.2, %B=0; t=22, %B=40) was run followed by a 3 min wash and 7 min equilibration. Flow was directed to an Agilent 6410 Triple Quadrupole MS (Santa Clara, CA) and source conditions

were set to 4000 V capillary voltage, 350 °C gas temperature, 12 L/min gas flow, and 30 psi nebulizer flow. All nucleotides were detected in negative ion MRM mode based on a characteristic fragmentation reaction (table 1). Quantitation of metabolites is based on the inclusion of isotope labeled internal standards and an external calibration using nucleotide standards in a mixed muscle matrix.

Table 1: Characteristic Fragmentation Reaction for Nucleotide Detection

Compound	Prec. (m/z)	MS1 Res	Prod. (m/z)	MS2 Res	Frag (V)	CE (V)	RT	Delta RT
Acadesine	257	wide	125	wide	110	15	1.2	3
Adenine	134	wide	107	wide	120	15	1.6	3
Guanosine	282	wide	150	wide	140	12	1.7	3
SAH	383	wide	134	wide	90	30	2.5	3
Adenosine	266	wide	134	wide	80	27	3	3
NAD	662.1	wide	540.1	wide	105	13	4.2	3
CMP	322	wide	211	wide	140	9	5.1	3
UMP	323	wide	111	wide	120	24	5.9	3
εNAD	686.1	wide	564.1	wide	115	15	6.7	3
UDP-galactose	565	wide	323	wide	160	24	6.7	3
GMP	362	wide	79	wide	95	25	6.9	3
IMP	347	wide	79	wide	120	39	7.1	3
GDP-mannose	604	wide	424	wide	160	30	7.4	3
AMP IS	361	wide	144	wide	80	37	9	3
13C10-AMP	356	wide	139	wide	80	37	9	3
AMP	346	wide	134	wide	80	37	9	3
ADP-ribose	558	wide	346	wide	160	30	9.5	3
MTA	296	wide	134	wide	80	12	9.5	3
XMP	363	wide	151	wide	95	30	11.4	3
CDP	402	wide	159	wide	130	27	11.5	3
NADH	664.1	wide	397.1	wide	130	33	11.6	3
UDP	403	wide	159	wide	110	27	12	3
GDP	442	wide	150	wide	140	25	12.4	3
NADP	742	wide	620.1	wide	120	15	12.7	3
13C10,15N5-ADP	441	wide	159	wide	130	27	14	3
13C10-ADP	436	wide	159	wide	130	27	14	3
ADP	426	wide	159	wide	130	27	14	3

13C9-CTP	491	wide	159	wide	160	33	15.9	3
CTP	482	wide	159	wide	160	33	15.9	3
dCTP	466	wide	159	wide	120	36	16	3
13C9-UTP	492	wide	159	wide	120	36	16.4	3
UTP	483	wide	159	wide	120	36	16.4	3
13C10-GTP	532	wide	434	wide	130	22	16.5	3
GTP	521.9	wide	424.1	wide	130	22	16.5	3
S-AMP	462	wide	134	wide	150	39	16.7	3
dGTP	506	wide	355	wide	130	18	17	3
dTTP	481	wide	159	wide	110	33	17.2	3
13C10,15N5-ATP	521	wide	159	wide	115	33	17.5	3
13C10-ATP	516	wide	159	wide	115	33	17.5	3
ATP	506	wide	159	wide	115	33	17.5	3
dATP	490	wide	392	wide	110	21	18.1	3
NADPH	371.6	wide	304.1	wide	105	10	18.4	3

2.13 Permeabilized Muscle Fiber Bundles (PmFB):

PmFB were prepared based on described methods (Anderson et al.2007, Perry et al.2011), which were adapted from previous studies (Tonkongi et al.2003, Kuznetsov et al.1996). Extracted muscle was placed in ice-cold biops buffer containing (in mM) 50MES, 7.23K₂EGTA, 2.77 CaK₂EGTA, 20 imidazole, 0.5DTT, 20 taurine, 5.7 ATP, and 6.56 MgCl₂-6 H₂O (pH 7.1, 290mOsm). The muscle was trimmed of connective tissue and fat and dissected into small portions. Small muscle bundles (2-7mm, 0.9-2.0 mg wet weight) were prepared from each mouse. Each bundle was gently separated along its longitudinal axis on ice with a pair of needle-tipped forceps under magnification (MX6 stereoscope, Leica Microsystems, Inc., Wetzlar, DE). Bundles were then incubated on a rotor with 40µg/mL saponin in biops buffer for 30 minutes at 4°C.

Saponin is a cholesterol-specific detergent that selectively permeabilizes the sarcolemmal membranes while keeping the mitochondrial membranes (which contain low levels of cholesterol), largely intact (Veksler et al.1987, Kuznetsov et al.2007). Fiber bundles were then washed for 10 minutes on a rotor at 4°C in respiration buffer containing (in mM) 105 K-MES, 30KCl, 1 EGTA, 10KH₂PO₄ and 5 MgCl₂-6 H₂O with 5mg/ml BSA (pH 7.4, 290 mOsm).

Mitochondrial oxygen consumption was measured in the OROBOROS Oxygraph-2k (OROBOROS Instruments, Corp., Innsbruck, AT). Fiber bundles were placed on blotting paper for 10 seconds and immediately weighed and transferred into the OROBOROS chamber containing 2mL respiration buffer and constant stirring at 750rpm. Experiments were run at 37°C, beginning with ~350µM O₂. Chambers were re-oxygenated if oxygen content fell below 300µM.

2.14 Exercise Studies:

Because of the inherent subjectivity of treadmill running to exhaustion, exercise studies were carried out in a blind fashion. Before exercise testing, mice were acclimated to the treadmill for three days prior to the study. Acclimation consisted of a three minute run with one minute at each of the first three speeds in the respective protocol being tested.

Endurance exercise was analyzed at a fixed slope of 10°. Mice began running at 8m/min. Treadmill speed was increased 2m/min every 15 minutes until

16m/min was attained. At this point, the treadmill was held at a consistent speed until exhaustion. Exhaustion was defined as remaining on the shocker plate for more than 10 seconds with nudging.

The transition from endurance to high intensity exercise protocol was run with a fixed slope of 10°. Similar to the endurance protocol described above, mice began running at 8 m/min and increased 2m/min every 15 minutes for 1 hour total. After the initial hour, the mice were transitioned into a higher intensity exercise with 1 minute at 20m/min, then 10 minutes at 23 m/min, with an increase of 3.5m/min every 10 min until exhaustion.

Exercise capacity during high intensity running was determined using an enclosed treadmill (Columbus instruments,) attached to the Comprehensive Laboratory Animal Monitoring System (CLAMS) with a fixed incline of 10°. 3-month old mice were run at 14 m/min with increasing speed by 3 m/min every 3 minutes until exhaustion. Measurements were collected every 30 seconds during which air flow was set to 0.6 l/min.

2.15 Isolated muscle Stimulation and Acetylcarnitine Oxidation Studies:

Braided silk suture loops were attached to muscle tendons and excised from the mouse. Muscles were placed in pre-warmed KHB buffer (pH 7.4) which contained (in mmol/l) 118 NaCl, 4.7 KCl, 2.52 CaCl₂, 1.64 MgSO₄, 24.88 NaCO₃, 1.18 KH₂PO₄, 5.55 glucose and 2.0 Na-pyruvate. After placement in the system,

muscles were tensed to 1 gram and allowed to relax for 15 minutes. Muscles were then re-tensed to 1 gram and allowed to rest 5 minutes. This tense/5 minute rest was repeated three times before initiation of stimulation. KHB buffer was used in muscle transport and tensing, while low glucose KHB (+2mM glucose, no pyruvate) was used during stimulation. The following stimulation protocol was used unless otherwise stated: stimulation rate: 60pps, delay: 2000ms, duration: 300ms, volts: 20 per chamber. O₂ flow and temperature (25°C) were kept constant throughout stretching and stimulation. Acetylcarnitine (Sigma; St. Louis, Mo), was made up at 1 M in H₂O and added at a final concentration of 5 mM to stimulating muscles 30 seconds after initiation of contraction. This allowed for normalization of contracting muscles prior to acetylcarnitine addition. Acetylcarnitine oxidation was assessed by capturing ¹⁴CO₂ from 200μM [1-¹⁴C]acetylcarnitine (ARC 1615) during rest or stimulation in a radnoti 2mL tissue bath system. Oxidation studies were done in low glucose KHB buffer with 3 mg/mL HEPES added.

2.16 Statistics:

JMP software version 7.0 (SAS Institute, Cary, NC) was used to perform multivariate correlation analyses of metabolites measured in Zucker diabetic fatty (ZDF) rats and lean controls. Other statistical analyses were performed using SigmaStat (SysStat Software, Inc., Point Richmond, CA) or the Microsoft Excel statistical package. Within-group responses to experimental manipulations were

evaluated using a paired t test, where appropriate. All data are presented as mean \pm S.E., and the level of significance was established *a priori* at p less than or equal to 0.05.

3 Obesity and Lipid Exposure Inhibit Carnitine Acetyltransferase Activity

Objective: Carnitine acetyltransferase (CrAT) is a mitochondrial matrix enzyme that catalyzes the interconversion of acetyl-CoA and acetylcarnitine. Recent studies have shown that enhanced or diminished forward flux through the CrAT reaction results in corresponding changes in total body glucose tolerance and muscle activity of PDH, a mitochondrial enzyme complex that regulates glucose oxidation. Because PDH activity is feedback inhibited by acetyl-CoA and negatively regulated by obesity and high fat feeding, we questioned whether overnutrition and/or lipid exposure might likewise diminish CrAT activity.

Methods: Tandem mass spectrometry-based metabolic profiling was used to investigate the relationship between muscle content of lipid intermediates and CrAT activity in multiple rodent models of obesity. Additionally, the direct effect of lipid exposure on CrAT activity was examined using purified enzyme, isolated mitochondria and human skeletal myocytes (HSkMC) grown in culture.

Results: CrAT specific activity was decreased in muscles from obese and diabetic rodents, despite increased protein abundance. This reduction in enzyme activity was accompanied by muscle accumulation of long chain acylcarnitines and acyl-CoAs, and a decline in the acetylcarnitine/acetyl-CoA ratio. *In vitro* assays of CrAT activity demonstrated that palmitoyl-CoA acts as a direct mixed-model inhibitor of CrAT, with an IC₅₀ ranging from 12 μ M to 178 μ M. Similarly,

in primary HSkMC grown in culture, nutritional and genetic manipulations that promoted mitochondrial influx of fatty acids resulted in accumulation of long chain acylcarnitines but a pronounced lowering of free carnitine and CrAT-derived short chain acylcarnitines.

Conclusions: These results show that nutrient overload inhibits CrAT activity and suggest that lipid-induced antagonism of this enzyme might contribute to decreased PDH activity and glucose disposal in the context of obesity and diabetes.

3.1 Introduction:

L-Carnitine is a conditionally essential nutrient that serves as a substrate for a family of acyltransferase enzymes that catalyze the interconversion of acyl-CoAs and acylcarnitines. Unlike their acyl-CoA precursors, acylcarnitines can be transported across cellular membranes. Accordingly, carnitine is best known for its obligatory role in shuttling long chain acyl-CoAs from the cytoplasm into the mitochondria matrix for fatty acid oxidation, a function that is mediated by the outer mitochondrial membrane enzyme, carnitine palmitoyltransferase 1 (CPT1). The long chain acylcarnitine products of CPT1 are transported across the inner mitochondrial membrane by carnitine acylcarnitine translocase and then converted back to long chain acyl-CoAs by carnitine palmitoyltransferase 2 (CPT2), also localized to the inner membrane. By contrast, carnitine acetyltransferase (CrAT) resides in the mitochondrial matrix and has strong

preference for short chain acyl-CoA intermediates of fatty acid, glucose and amino acid catabolism. Thus, CrAT facilitates trafficking and efflux of carbon intermediates from the mitochondrial compartment to other cellular and extracellular sites.

Recent animal studies have established important roles for L-carnitine and CrAT in regulating glucose homeostasis and mitochondrial substrate switching (Muoio et al. 2012). By converting acetyl-CoA to acetylcarnitine, CrAT not only buffers the mitochondrial acetyl-CoA pool but also regenerates free CoA, both of which influence the activities of several oxidative enzymes. Dietary L-carnitine supplementation administered to obese or diabetic rodents promotes acetylcarnitine efflux and encourages carbon flux through pyruvate dehydrogenase (PDH; Noland et al. 2009), the enzyme complex that connects glycolysis to glucose oxidation and which is feedback inhibited by its product, acetyl-CoA. Fitting with the notion that CrAT mitigates acetyl-CoA inhibition of PDH, mice with muscle-specific deletion of *crat* show impaired switching from fatty acid to glucose-derived fuels during the fed-to-fasted transition (Muoio et al. 2012). These perturbations in fuel metabolism were associated with intramuscular accumulation of short, medium and long chain acyl-CoAs, decreased PDH activity and development of whole body insulin resistance (Muoio et al. 2012).

Because PDH activity, substrate switching and glucose tolerance are negatively impacted by obesity and high fat feeding, the present study sought to determine whether these nutritional and pathophysiological conditions might likewise impinge upon CrAT activity. To this end, we examined changes in acylcarnitine/acyl-CoA balance, CrAT expression and CrAT activity in a variety of rodent and cell culture models of nutrient-induced metabolic dysfunction. We found that CrAT activity was indeed decreased in response to genetic diabetes, high fat feeding and lipid exposure. Taken together with previous studies, these results suggest that diminished CrAT activity might contribute to low PDH activity and impaired glucose disposal in the context of obesity and diabetes.

3.2 Materials and Methods

Animals. Animal studies were approved by the Duke University Institutional Animal Care and Use Committee. Male Wistar rats (150-175 g, Charles River) were single housed and allowed *ad libitum* access to food and water. Animals were randomly selected to receive twenty weeks of either a low fat diet (D12450B) or a 45% high fat diet (D12451; Research Diets) beginning at 3 months of age. Male Zucker diabetic fatty (ZDF) rats and lean controls (Charles River) were allowed *ad libitum* access to standard chow and water before harvest at 3 months of age. Rats were sacrificed after intraperitoneal injection of

Nembutal with the dose of 25 mg/kg body weight. Gastrocnemius samples were clamp frozen and stored at -80°C. Tissues were ground into powder and processed in CellLytic buffer (Sigma Chemicals, St. Louis, MO) by freeze fracturing three times and sonication at five times one second pulses on setting five.

Mitochondrial Isolation. Skeletal muscle mitochondria were prepared according to (Kerner et al. 2001) with modification. Mouse gastrocnemius muscles were removed under anesthesia and placed in ice cold KMEM buffer (100mM KCl, 50 mM MOPS, 1 mM EGTA, 5 mM MgSO₄, pH 7.4). The tissue was cleaned, blotted, weighed, finely minced, and suspended at a 10-fold dilution in KMEM plus 1mM ATP. The suspension was homogenized on ice using ten passes with a Potter-Elvehjem homogenizer. KMEM/ATP buffer supplemented with 0.2% BSA was then added to achieve a 20- fold dilution. The homogenate was centrifuged at 500xg for 10 min at 4 °C. The supernatant was then centrifuged at 10,000xg for 10 minutes at 4 °C and the pellet was resuspended in 500µL of KMEM/ATP buffer and centrifuged at 7000xg for 10 min at 4 °C to wash. The pellet was resuspended in 500uL KMEM and centrifuged at 7000xg for 10 min at 4 °C. The resulting mitochondrial pellet was resuspended in CellLytic lysis buffer (Sigma Chemicals, St. Louis, MO) and processed by freeze fracturing three times and sonication at five times one second pulses on setting five.

Carnitine acetyltransferase activity. Carnitine-dependent conversion of acetyl-CoA to acetylcarnitine and free CoA was measured as previously described with minor modifications (Muoio et al.2012). Acetyl-CoA and 0.1 mM DTNB [5,5'dithiobis(2-nitrobenzoic acid)] were combined with purified enzyme, cell lysates or isolated mitochondria. The assay buffer included 50 mM Tris and 1 mM EDTA in water at pH 7.8. CrAT activity was determined spectrophotometrically at 412 nm by evaluating the rate of reduction of DTNB by free CoA on a Spectramax M5 spectrophotometer (Molecular Devices). Samples were read for 2 minutes in the absence of carnitine to determine a baseline rate. Reactions were started with the addition of 5 mM L-carnitine (unless stated otherwise) and monitored every 20 seconds for 10 minutes. The 2 minute baseline rate was then subtracted from the final linear rate to yield a corrected rate. Activity calculations were made using an instrument specific extinction coefficient for TNB of $16,029 \text{ M}^{-1}\text{cm}^{-1}$ determined using L-glutathione as a CoA donor and correcting for a path length of 0.641 cm (for a 0.2 ml reaction volume in NUNC 96-well plates). All substrates were purchased from Sigma and reconstituted in activity buffer.

Western blots. Protein was isolated using CellLytic lysis buffer (Sigma Chemicals, St. Louis, MO). A bicinchoninic acid (BCA) kit (Sigma Chemicals, St. Louis, MO)

was used to quantify protein. Protein (50µg from cell/tissue lysates) was separated by SDS-PAGE, transferred to nitrocellulose, and incubated with antibodies prepared with 5% milk in TBS-Tween. Secondary antibodies were HRP-conjugated and ECL detection reagent (Pierce, Rockford, IL) was used to visualize protein bands. Protein expression was normalized to total protein as determined by MemCode staining (Thermo Scientific). The primary CrAT antibody was a generous gift from the laboratory of Dr. Fausto Hegardt.

Metabolic profiling. Tissue and plasma samples were processed and analysed by the Sarah W. Stedman Nutrition and Metabolism metabolomics/biomarker core laboratory. Acylcarnitine measurements were made using flow injection tandem mass spectrometry and sample preparation methods described in (An et al. 2004, Haqq et al. 2005). Acyl-CoA esters were analyzed using a method based on a previously published report (Magnes et al. 2005) which relies on the extraction procedure described by (Deutsch et al. 1994). The CoAs were further purified by solid phase extraction as described by (Minkler et al. 2008).

Cell Culture. Primary human skeletal muscle myocytes (HskMC) were grown and differentiated as previously described (Muoio et al. 2002), but with the addition of 100µM L-carnitine in the differentiation medium. Cytomegalovirus (CMV) promoter-driven recombinant adenoviruses (rAd) encoding either β-

galactosidase (rAd- β -gal) or Myc-tagged rat carnitine palmitoyltransferase b (rAd-CPT-1) were constructed, amplified and purified as previously described (Becker et al. 1994). On differentiation day 3, myotubes were treated overnight with 5.3×10^{-3} infectious units/cm² rAd- β -gal or rAd-CPT-1. Medium was replaced on differentiation day 4. On day 6, cells were treated with differentiation medium containing 1% BSA, alone or complexed with 100 μ M or 500 μ M 1:1 oleate:palmitate, along with 1 mM L-carnitine. Conditioned culture medium and cell lysates were harvested on differentiation day 7. Cells were washed twice with PBS, flash frozen in liquid nitrogen and lysates were harvested in water after scraping. Specimens were submitted to the Stedman Metabolomics Core Laboratory for profiling of acylcarnitines. Metabolite concentrations were normalized to total cellular protein. Similarly, rAD-CrAT treatment and processing was done according to (Noland et al. 2009).

Statistics. Statistical analyses were performed using SigmaStat (SysStat Software, Inc., Point Richmond, CA) or the Microsoft Excel statistical package. Within-group responses to experimental manipulations were evaluated using a paired *t* test, where appropriate. IC₅₀ values were calculated using Prism Graphpad software. Data are presented as means \pm S.E., and the level of significance was established *a priori* at *p* less than or equal to 0.05.

3.3 Results

Obesity and Diabetes Disrupt Acyl-CoA Buffering: Obesity and diabetes are associated with muscle accumulation of medium and long chain acylcarnitines (LCAC) (Noland et al. 2009, Koves et al. 2008). Because these intermediates are presumed to be in equilibrium with their cognate acyl-CoA precursors (Brass et al. 1980, Carter et al. 1981), here we sought to examine tissue fluctuations in these two interconnected metabolite pools. As expected, LCAC and long chain acyl-CoAs were increased (up to 4.6 fold) in both heart and gastrocnemius muscles from Zucker diabetic fatty (ZDF) rats compared to lean controls, and to a lesser degree from those fed a high fat (HF) diet versus a low fat (LF) control diet (Table 2 and Figure 6). Despite pronounced accumulation of LCAC and MCAC species in heart and muscle of diabetic and/or obese rodents, acetylcarnitine (C2) levels were unchanged and tended to decrease in these same tissues (Table 2). Muscle levels of acetyl-CoA, the principal substrate of CrAT, were unchanged in the diabetic model but increased 1.8-fold in gastrocnemius muscles from rats fed a high fat versus low fat diet.

Table 2: Obesity and Diabetes Disrupt Acyl-CoA Buffering.

Tandem mass spectrometry was used to assess total long chain acylcarnitines (LCAC), long chain acyl-CoAs (LCACoA), medium chain acylcarnitines (MCAC), medium chain acyl-CoAs (MCACoA), acetylcarnitine and acetyl-CoA. Metabolites were measured in tissue homogenates from obese Zucker diabetic *fatty* (ZDF) rats and lean control animals, and from adult Wistar rats fed a 10% low fat (LFD) or 45% high fat diet (HFD) for twenty weeks. Data are expressed as pmol/mg tissue and represent means \pm S.E. from 5-8 animals per group. * $p < 0.05$, lean vs. obese.

	Heart		Gastrocnemius Muscle		Gastrocnemius Muscle	
	Lean	ZDF	Lean	ZDF	LFD	HFD
Total LCAC (pmol/mg tissue)	7.7 \pm 2.4	35.6 \pm 7.1*	8.3 \pm 1.5	28.6 \pm 3.5*	2.3 \pm 0.5	3.4 \pm 0.6
Total LCACoA (pmol/mg tissue)	12.6 \pm 3.6	41.4 \pm 5.8*	5.0 \pm 0.3	11.2 \pm 0.9*	7.2 \pm 0.4	11.2 \pm 1.0*
Total MCAC (pmol/mg tissue)	0.6 \pm 0.2	2.0 \pm 0.4*	1.1 \pm 0.2	3.5 \pm 0.4*	0.6 \pm 0.2	0.7 \pm 0.1
Total MCACoA (pmol/mg tissue)	5.3 \pm 0.6	6.3 \pm 0.5	1.6 \pm 0.2	1.9 \pm 0.1	2.0 \pm 0.2	2.1 \pm 0.2
Acetylcarnitine (pmol/mg tissue)	181.6 \pm 16.3	135.6 \pm 16.3	153.0 \pm 10.7	153.6 \pm 8.8	76.6 \pm 12.2	54.4 \pm 7.4
Acetyl-CoA (pmol/mg tissue)	15.4 \pm 1.4	16.0 \pm 0.5	1.8 \pm 0.2	2.2 \pm 0.07	0.58 \pm 0.02	1.0 \pm 0.07*

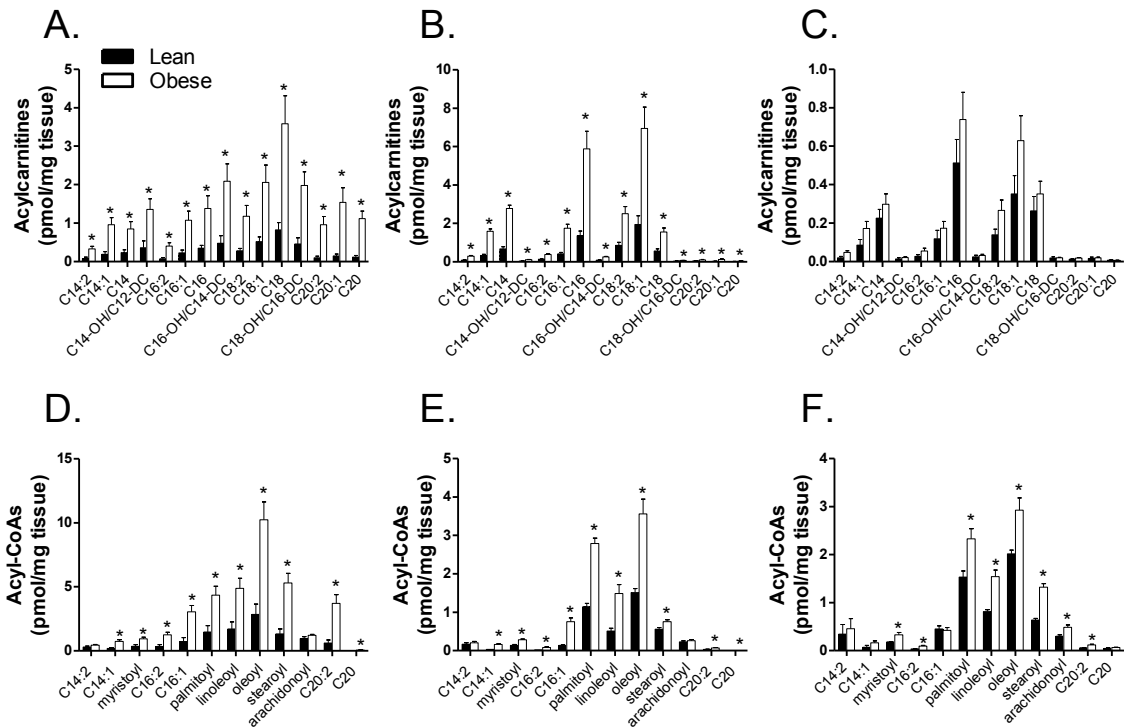


Figure 6: Long Chain Acylcarnitine and Acyl-CoA Profiling of Muscle from Obese and Diabetic Rats.

Tandem mass spectrometry was used to assess long chain acylcarnitines (A-C) and long chain acyl-CoAs (D-E) in tissue homogenates prepared from lean (filled bars) and obese (open bars) rats. Acylcarnitines measured in (A) heart and (B) gastrocnemius muscles from obese Zucker diabetic *fatty* (ZDF) rats and lean controls; and (C) gastrocnemius muscles from Wistar rats fed a 10% low fat or 45% high fat diet for twenty weeks. Acyl-CoAs measured in (D) heart and (E) gastrocnemius muscles from obese Zucker diabetic *fatty* (ZDF) rats and lean controls; and (F) gastrocnemius muscles from Wistar rats fed a 10% low fat or 45% high fat diet for eight weeks. Data represent means \pm S.E. from 5-8 animals per group. * $p < 0.05$ lean vs. obese.

CrAT specific activity is diminished in rodent models of obesity and diabetes. Collectively, the results in Table 2 revealed discordant fluctuations in the myocellular acyl-CoA and acylcarnitine pools in response to obesity and diabetes. Figure 7A highlights the obesity-associated shift in the

acetylcarnitine/acetyl-CoA ratio, which decreased 23 and 58% in muscles from the ZDF and HF fed rats, respectively, as compared to controls. Because a similar imbalance resulted from muscle-specific deletion of *crat* (Muoio et al. 2012), we questioned whether these observations might be indicative of reduced enzyme activity. To test this possibility we first measured changes in CrAT mRNA and protein expression in the same models. Interestingly, CrAT mRNA levels tended to increase (Figure 7B) whereas CrAT protein abundance (Figure 7C and D) was doubled in gastrocnemius muscles from ZDF compared to lean control rats. Likewise, the obesogenic HF diet did not affect CrAT mRNA but increased protein abundance by 24% compared to the LF diet controls (Figure 7B-D). Despite the 2-fold rise in CrAT protein levels measured in tissue lysates from ZDF compared to control rats, CrAT activity (measured in homogenates prepared from the same tissues) was elevated by only 20% (Figure 7E). Similarly, CrAT activity was unchanged in homogenates prepared using muscles from the HF-fed rats, despite a 24% increase in CrAT protein levels (Figure 7E). Thus, when CrAT activity was corrected for protein abundance, specific activity of the enzyme was reduced 30% and 26%, in the ZDF and HF diet models, respectively (Figure 7F). Similar results were observed in heart homogenates from ZDF compared to lean control rats (Figure 8) and skeletal muscle homogenates of C57BL6/J mice fed a HF compared to a LF diet (Figure 9). Thus, this response is consistent across tissues and species.

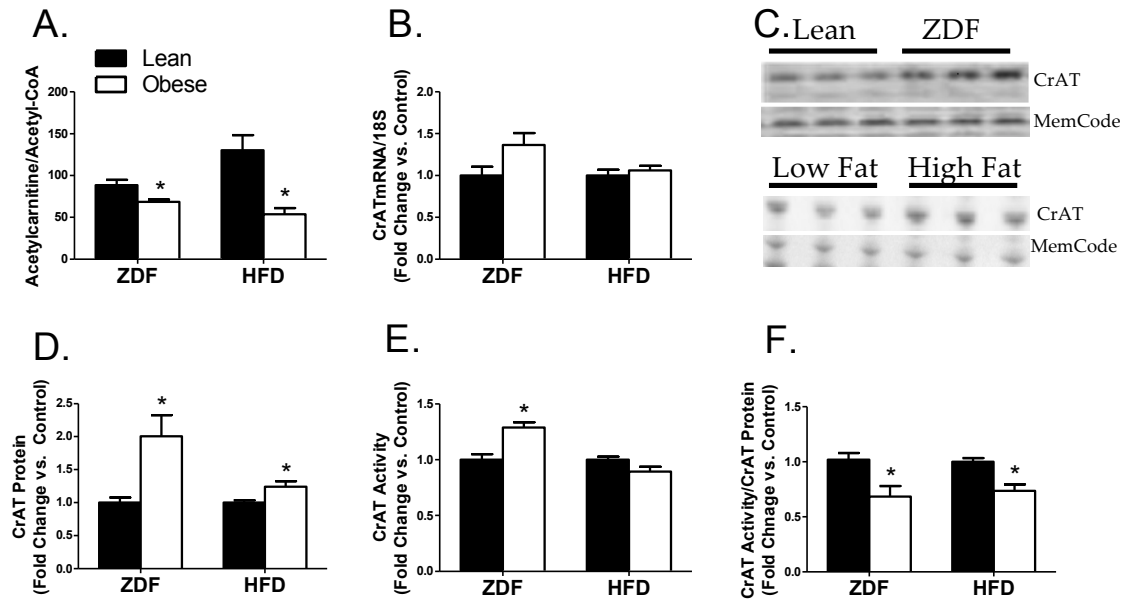


Figure 7: CrAT Specific Activity is Diminished by Obesity and Diabetes.

Gastrocnemius muscles were harvested from obese Zucker diabetic *fatty* (ZDF) rats and lean control animals, and from adult Wistar rats fed a 10% low fat or 45% high fat diet for twenty weeks. (A) Tandem mass spectrometry-based measurement of the acetylcarnitine:acetyl-CoA ratio. (B) CrAT mRNA expression normalized to 18S. (C) Representative western blots and (D) protein abundance of CrAT normalized to the MEMCode stain. (E) Total CrAT enzyme activity and (F) CrAT specific activity corrected for CrAT protein abundance. Data are expressed as fold change relative to the lean control group and represent means \pm S.E. of 5-8 animals per group. * $p < 0.05$ lean vs. obese.

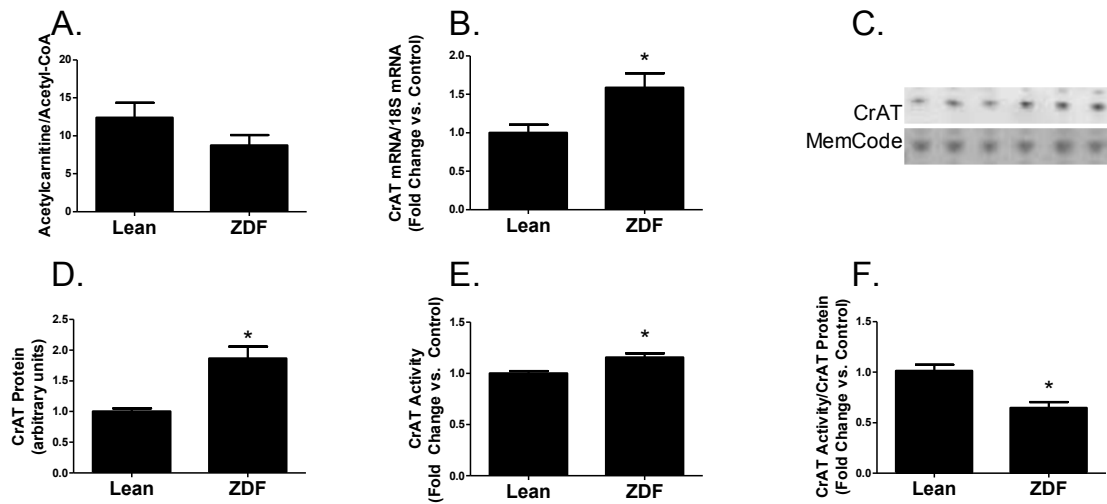


Figure 8: CrAT Specific Activity is Diminished in the Diabetic Heart.

Hearts were harvested from obese Zucker diabetic *fatty* (ZDF) rats and lean control animals. (A) Tandem mass spectrometry-based measurement of the acetylcarnitine:acetyl-CoA ratio. (B) CrAT mRNA expression normalized to 18S. (C) Representative western blots and (D) protein abundance of CrAT normalized to the MEMCode stain. (E) Total CrAT enzyme activity and (F) CrAT specific activity corrected for CrAT protein abundance. Data are expressed as fold change relative to the lean control group and represent means \pm S.E. of 5-8 animals per group. * $p < 0.05$ lean vs. ZDF.

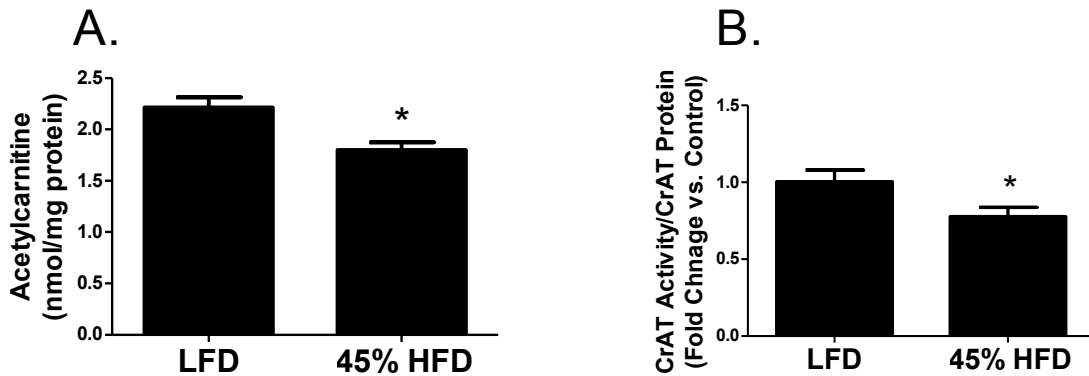


Figure 9: High Fat Feeding Decreases CrAT Specific Activity in Mouse Muscle.

Gastrocnemius muscles were harvested from mice fed a 10% low fat (LFD) or 45% high fat diet (HFD) for 4 weeks and used for (A) tandem mass spectrometry-based measurement of acetylcarnitine content, and (B) assessment of CrAT specific activity corrected for CrAT protein abundance. Data are expressed as fold change relative to the lean control group and represent means \pm S.E. of 5-8 animals per group. * $p < 0.05$ 10% vs. 45% HFD.

Long chain acyl-CoAs inhibit CrAT activity. Results in Figure 7 revealed a negative relationship between intramuscular lipid accumulation and CrAT activity. Relevant to this observation, earlier studies reported that long chain acyl-CoAs inhibited the activity of purified pigeon CrAT (Chase et al. 1967, Huckle et al. 1983, Mittal et al. 1980). Here, we sought to examine the inhibitory actions of palmitoyl-CoA on the activity of rat and mouse CrAT when assayed in a more native environment. To this end, we first compared CrAT substrate preference and enzyme kinetics using purified pigeon protein (Figure 10A), lysates of HSkMC treated with a rAd encoding rCrAT (Figure 10B), and isolated

mitochondria from mouse gastrocnemius muscle (Figure 10C). Consistent with earlier reports, both pigeon and rodent CrAT strongly preferred short chain acyl-CoAs and had essentially no activity with palmitoyl-CoA. Preference for specific short chain substrates differed by species. Whereas purified pigeon CrAT preferred propionyl-CoA>acetyl-CoA>butyryl-CoA, mouse and rat CrAT had greatest preference for butyryl-CoA, followed by propionyl-CoA>acetyl-CoA (Figure 10B and C). Affinity for L-carnitine was similar in the three systems assayed, each showing a K_m of approximately 0.10 mM (Figure 10D)

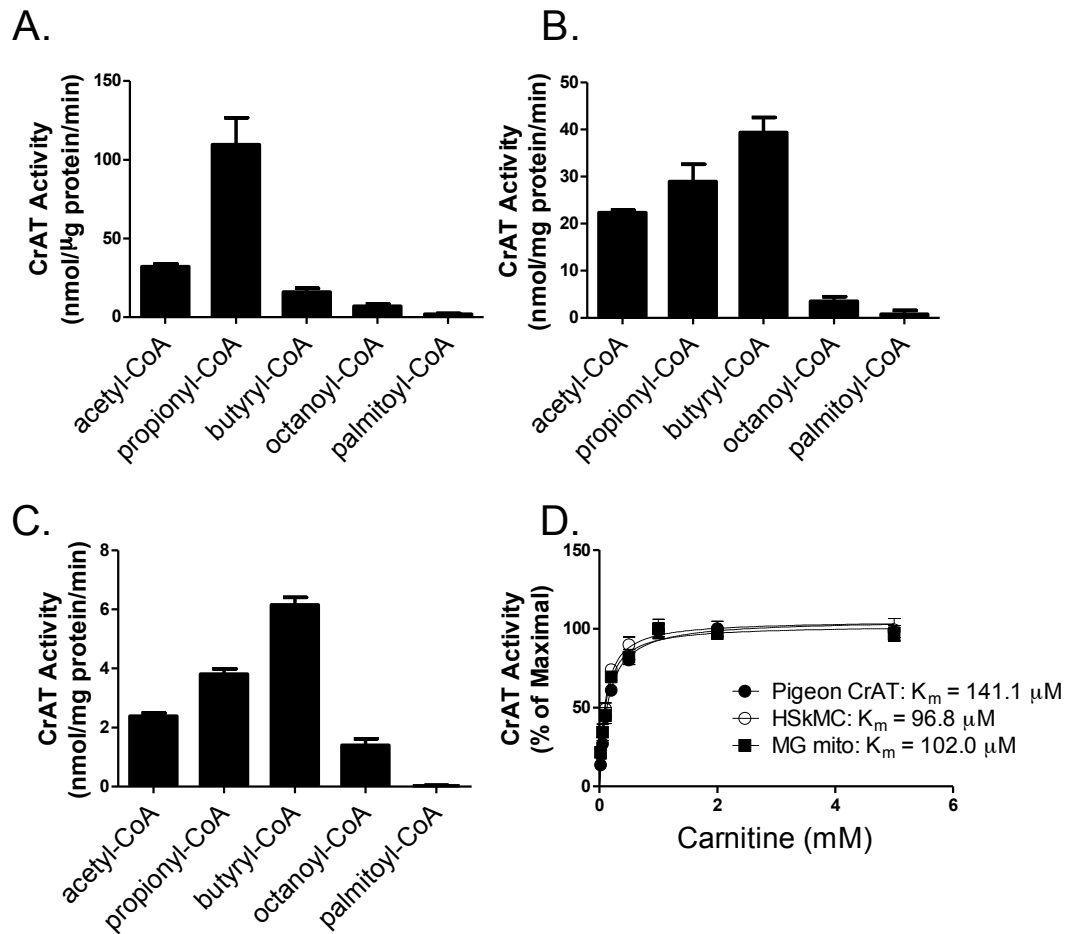


Figure 10: CrAT Prefers Short Chain Acyl-CoAs.

CrAT enzyme activity was measured with 0.45 mM acyl-CoA substrates of various chain lengths and 5.0 mM L-carnitine using (A) purified CrAT from pigeon breast muscle, (B) lysates from primary human skeletal muscle myocytes (HskMC) expressing recombinant rat CrAT and (C) isolated mitochondria from mouse gastrocnemius muscle (MG). (D) CrAT activity as a function of increasing carnitine concentration measured in the presence of 0.45mM acetyl-CoA. Results are expressed as percent of maximal CrAT activity. Data represent means \pm S.E. from 3-5 separate experiments.

We next examined palmitoyl-CoA inhibition of CrAT activity using the same three systems. Palmitoyl-CoA inhibited purified pigeon CrAT with an IC50

of 30.6 μM (Figure 11A), whereas the IC_{50} s for rat and mouse CrAT measured in HSkMC lysates and isolated mitochondria were 178 μM and 65 μM , respectively (Figures 11B and E). Oleoyl-CoA inhibited CrAT activity in a similar manner (data not shown). In isolated mitochondria, 40-60 μM palmitoyl-CoA reduced the V_{max} of CrAT by 11- 23% (Figure 11C). Intersection of the double reciprocal (Lineweaver-Burk) plots occurred to the left of the vertical ($1/v$) axis (Figure 11D), reflecting a mixed model form of inhibition. This result implies a combination of uncompetitive inhibition (inhibitor binds only to the enzyme-substrate complex) and competitive inhibition (inhibitor binds only in the absence of substrate). Thus, palmitoyl-CoA appears to bind CrAT regardless of whether short chain acyl-CoA substrate is already bound. Interestingly however, the concentration of palmitoyl-CoA necessary for half-maximal inhibition of CrAT increased from 12 μM to 65 μM in the presence of low (0.05 mM) as compared to high (2 mM) carnitine concentrations (Figure 11E). This implies that binding of carnitine to CrAT reduces its affinity for palmitoyl-CoA, suggesting that long chain acyl-CoAs function as more potent inhibitors of the enzyme when mitochondrial concentrations of free carnitine are low.

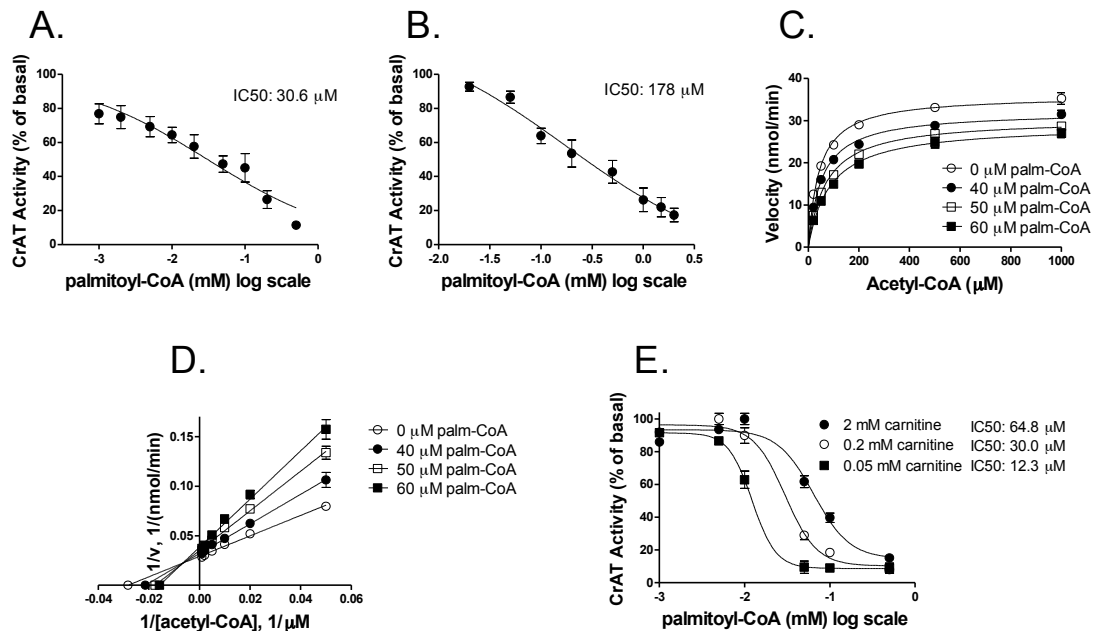


Figure 11: Long Chain Acyl-CoAs Inhibit CrAT Activity.

CrAT activity as a function of increasing palmitoyl-CoA concentration was measured using (A) purified protein from pigeon breast, (B) isolated mitochondria from primary human skeletal myocytes (HskMC) expressing recombinant rat CrAT and (C, D) isolated mitochondria from mouse gastrocnemius muscle. Assays in panels A, B and E were performed with 0.45 mM acetyl-CoA and 5 mM L-carnitine. (E) Palmitoyl-CoA inhibition of CrAT activity as a function of L-carnitine concentration using isolated mitochondria from mouse gastrocnemius. Data represent means \pm S.E. from 3-5 separate experiments (A,B), or 5-8 animals per group (C-E).

Lipid exposure and increased CPT1 activity reduced acetylcarnitine production in primary human skeletal myocytes. We next sought to determine whether chronic exposure of muscle mitochondria to elevated fatty acids is sufficient to inhibit CrAT activity in an intact cellular system. To this end, primary HskMC were incubated with increasing doses of a 1:1 mixture of oleate:palmitate and short chain acylcarnitine profiles were used as a surrogate index of CrAT activity. Additionally, to promote fatty acid influx into the mitochondrial matrix

where CrAT resides, HSkMC were also treated with recombinant adenovirus encoding rat CPT1b. This experimental paradigm was designed to mimic both the lipid conditions and increased expression of CPT1b that are characteristic of obesity and diabetes (Zou et al. 2009).

After 24 h exposure to fatty acids, control cells treated with rAd encoding β -galactosidase (β -gal) increased production of palmitoylcarnitine (C16), 3-hydroxypalmitoylcarnitine (C16:OH) and lauroylcarnitine (C:12) in a dose-dependent fashion (Figures 12A-C). The C12 and C16:OH carnitine species are generated as byproducts of beta-oxidation. Thus, cellular accumulation of these intermediates is likely to reflect increased production of the respective acyl-CoA precursors within the mitochondrial compartment. Compared to the rAD- β -gal control group, treatment of cells with rAd-CPT1b caused a 4 to 9-fold increase in cellular levels of palmitoylcarnitine (C16), the direct product of the enzyme. Overexpression of rAd-CPT1b also elevated most long and medium chain acylcarnitines, suggesting increased mitochondrial transport and catabolism of fatty acids (Figures 12A-C). Since medium and long chain acylcarnitines are thought to be in equilibrium with their acyl-CoA counterparts, these results support the notion that lipid exposure, especially in combination with CPT1b overexpression, leads to a rise in the mitochondrial pool of long chain acyl-CoAs.

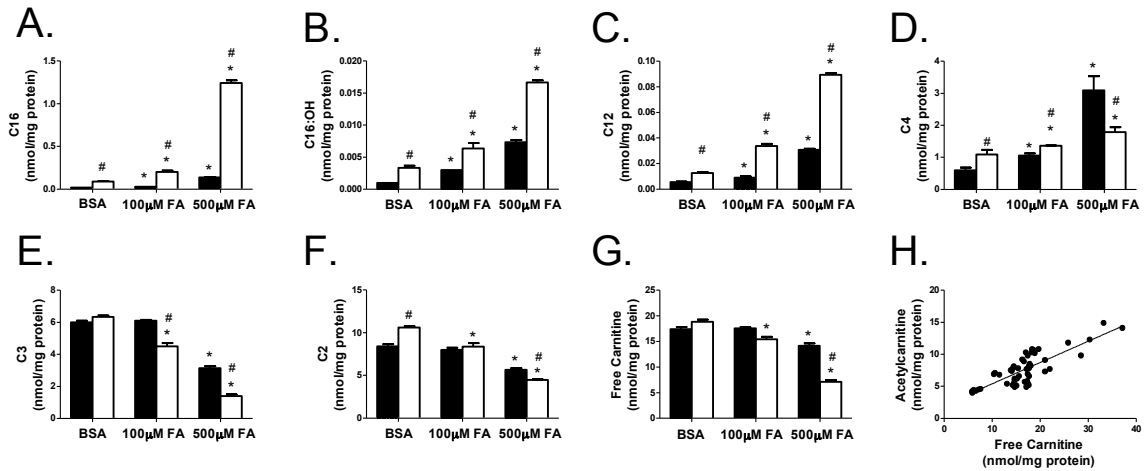


Figure 12: Fatty Acid Exposure and CPT1 Overexpression Decrease Short Chain Acylcarnitine Production in Human Skeletal Myocytes

Acylcarnitine levels were measured in lysates prepared from primary human skeletal myocytes treated with recombinant adenovirus encoding β -galactosidase (filled bars) or rat carnitine palmitoyltransferase-b (CPT1b) (open bars). Cells were treated with adenoviruses on differentiation day 3 and harvested on day 7 following 24 h exposure to 1% BSA alone or complexed with 100 or 500 μ M 1:1 oleate:palmitate, along with 1 mM L-carnitine. Specific acylcarnitine species shown include, (A) palmitoylcarnitine (C16), (B) 3-hydroxypalmitoylcarnitine (C16:OH), (C) lauroylcarnitine (C12), (D) butyrylcarnitine (C4), (E) propionylcarnitine (C3), (F) acetylcarnitine (C2) and (G) free carnitine. (H) Cellular levels of free carnitine and acetylcarnitine were strongly correlated. * $p < 0.05$ fatty acid treatment vs. BSA control, # $p < 0.05$ rAd-CPT1b vs. β -gal control virus.

Interestingly, despite marked lipid-induced increases in long and medium chain acylcarnitines, a disparate pattern emerged for the short chain products of the CrAT reaction. For example, cellular accumulation of butyrylcarnitine (C4) increased dose-dependently in the control cells but not the rAd-CPT1b treated group. On the contrary, conditions of high lipid exposure (500 μ M O:P) caused C4 levels to decrease 50% in the CPT1b overexpressing cells as compared to controls (Figure 12D). Moreover, fatty acid exposure resulted in a dose-dependent decrease in both propionylcarnitine (C3) and acetylcarnitine; and this

effect was exacerbated by CPT1b overexpression (Figure 12E-G). A nearly identical pattern emerged when we measured acylcarnitine efflux into the culture medium during the same 24 h period of lipid exposure (Figure 13). Interestingly, despite ample provision of extracellular carnitine (1 mM), intracellular content of free carnitine declined in response to lipid exposure, particularly in the rAd-CPT1b group. Also noteworthy, cellular concentrations of free carnitine correlated strongly with acetylcarnitine (Figure 12H) but not LCAC (not shown). These observations are consistent with the notion that lipid exposure antagonizes CrAT activity, not only by increasing mitochondrial concentrations of long chain acyl-CoAs but also by reducing availability of free carnitine within the mitochondrial matrix (Figure 14).

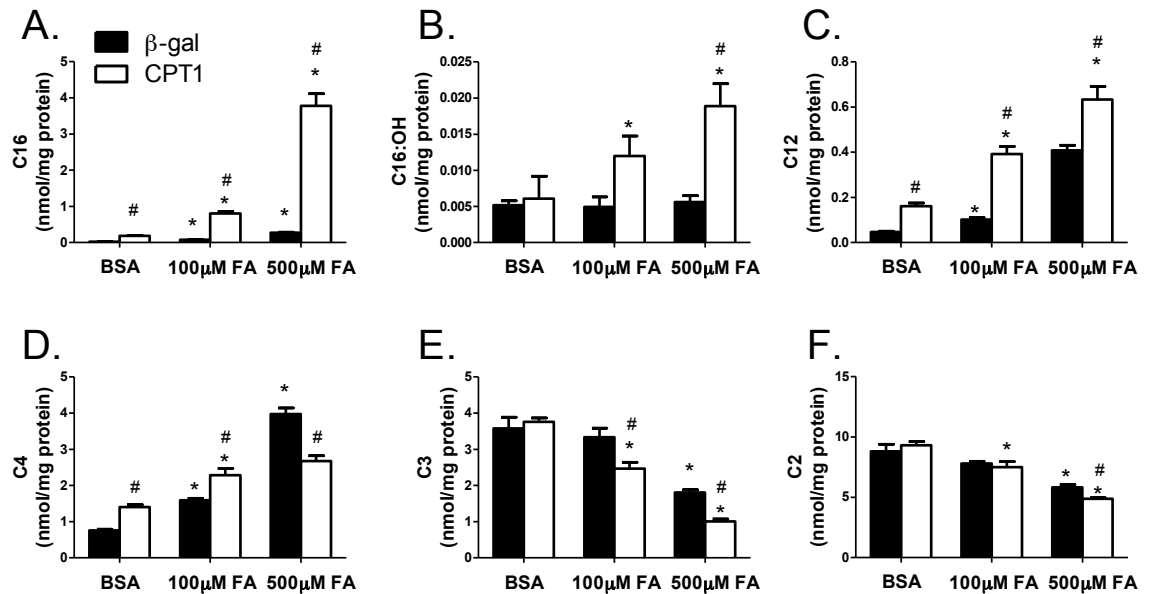


Figure 13: Fatty Acid Exposure and CPT1 Overexpression Decrease Short Chain Acylcarnitine Efflux from Human Skeletal Myocytes.

Acylcarnitine levels were measured in culture media from primary human skeletal myocytes treated with recombinant adenovirus encoding β -galactosidase (filled bars) or rat carnitine palmitoyltransferase- β (CPT-1 β) (open bars). Cells were treated with adenoviruses on differentiation day 3 and harvested on day 7 following 24 h exposure to 1% BSA alone or complexed with 100 or 500 μ M 1:1 oleate:palmitate, along with 1 mM L-carnitine. Specific acylcarnitine species shown include, (A) palmitoylcarnitine (C16), (B) 3-hydroxypalmitoylcarnitine (C16:OH), (C) lauroylcarnitine (C12), (D) butyrylcarnitine (C4), (E) propionylcarnitine (C3) and (F) acetylcarnitine (C2). * $p < 0.05$ fatty acid treatment vs. BSA control, # $p < 0.05$ rAd-CPT1b vs. β -gal control virus.

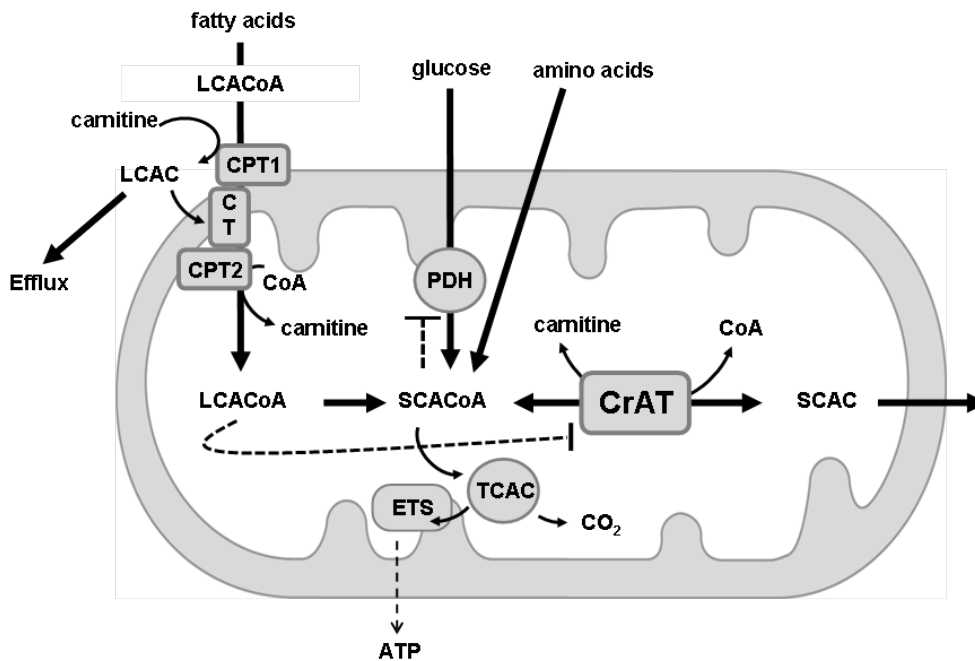


Figure 14: Proposed Model of Lipid-Induced Inhibition of CrAT Activity.

Fatty acid-derived long chain acyl-CoAs (LCACoA) cross the mitochondrial double membrane via the carnitine/acylcarnitine transport system. After traversing the inner membrane through the action of carnitine acylcarnitine translocase (CT), the acylcarnitine products of CPT1 are converted back to LCACoAs by CPT2, thereby replenishing the matrix pool of free carnitine. Carnitine acetyltransferase (CrAT) converts short chain acyl-CoA (SCACoA) intermediates of fat, glucose and amino acid metabolism to short chain acylcarnitine (SCAC) metabolites that can efflux from the mitochondria when nutrient supply and catabolism exceeds flux through the tricarboxylic acid cycle (TCAC) and the electron transport system (ETS). CrAT-mediated lowering of acetyl-CoA disinhibits pyruvate dehydrogenase (PDH), promoting glucose oxidation. Excessive formation and cellular efflux of LCAC depletes the matrix pool of free carnitine, thereby limiting CrAT activity. Additionally, LCACoAs act as direct allosteric inhibitors of CrAT. These mechanisms might serve to counter-regulate the activities of CrAT and PDH to spare glucose and promote beta-oxidation when lipids are plentiful.

3.4 Discussion:

CrAT regulates the intra-mitochondrial pool of short chain acyl-CoAs. Because emerging evidence indicates this enzyme plays an essential role in regulating whole body glucose homeostasis, the present investigation sought to determine whether nutritional stresses that perturb metabolic control likewise alter CrAT expression and/or function. Our study produced three major findings. First, in skeletal muscle and heart from obese rodents, tissue balance between acetylcarnitine and acetyl-CoA was disrupted in accord with reduced CrAT specific activity and elevated concentrations of long chain acyl-CoAs. Second, we confirmed earlier reports that CrAT activity is potently inhibited by long chain acyl-CoAs. Lastly, experiments in primary human myocytes showed that nutritional and genetic manipulations that promote fatty acid delivery to mitochondria caused a marked decline in cellular production and efflux of CrAT-derived acylcarnitines. In aggregate, these results suggest that CrAT function is compromised by chronic overnutrition, due at least in part to lipid-induced inhibition of enzyme activity.

Chronic overnutrition results in accumulation of triacylglycerol and other lipid-derived metabolites in multiple tissues (Goodpaster and Kelley 2002, Muoio and Newgard 2008, Haqq et al. 2005). Consistent with these reports, the current study observed an increase in medium and long chain acyl-CoA metabolites in obese and diabetic rodents. Because β -oxidation is a rich source of short chain

acyl-CoAs, and because CrAT acts on acetyl-CoA derived from both glucose and fat catabolism (Muoio et al. 2012, Uziel et al. 1988), we anticipated that muscle acetyl-CoA and acetylcarnitine concentrations would rise in concert with long chain metabolites during periods of nutrient surplus. Instead, muscle acetylcarnitine levels remain unchanged or diminished while acetyl-CoA levels tended to increase (table 2). The corresponding decline in the acetylcarnitine/acetyl-CoA ratio was accompanied by decreased CrAT specific activity in each of the three models of obesity examined in this study (Figure 7-9).

CrAT catalyzes the reversible reaction between short chain acyl-CoA and acylcarnitine with an equilibrium constant of 1.5–1.8 (Farrell et al. 1984, Pieklik and Guynn 1975). Therefore, enzyme activity has long been thought to be regulated by substrate/product concentrations within the mitochondrial matrix. However, we found that obesity and diabetes lowered CrAT specific activity at a post-translational level. Because early studies showed that LCACoAs can inhibit purified preparations of pigeon, rat and bovine CrAT (Chase et al. 1967, Huckle et al. 1983, Mittal et al. 1980), we assessed this form of regulation in a more native enzyme environment. Using isolated muscle mitochondria and muscle cell lysates, we found that palmitoyl-CoA acts as a potent inhibitor of CrAT activity with an IC₅₀ of 12-65 μ M. Likewise, fatty acid exposure on HSkMC resulted in decreased cellular production and efflux of short chain acylcarnitine species, especially when CPT1b was overexpressed. Importantly, fatty acid exposure and

or CPT1b overexpression resulted in diminished levels of CrAT-derived substrates only, with medium and long chain acylcarnitines changing in the opposite direction (Figure 13). In aggregate, these findings support the hypothesis that accumulation of long chain acyl-CoAs leads to a reduction in CrAT activity. Though the foregoing *in vitro* assays point to allosteric inhibition as a potential mode of regulation, CrAT activity might alternatively be regulated via covalent modification. Protein acylation is a post translational form of enzyme regulation caused by reversible binding of an acyl-CoA group to one or more cysteine residues. Acylation regulates multiple enzymes involved in glucose metabolism including GAPDH (Yang et al. 2005) and phosphofructokinase-1 (Jenkins et al. 2011). Once acylated, these enzymes have decreased activity leading to a decline in glucose metabolism, supporting the Randle hypothesis or the lipid-mediated lowering of glucose oxidation (Randle et al. 1963). The mechanism by which acylation occurs is not clear. Acylation can result from the enzymatic action of palmitoyl acyltransferase (Dunphy et al. 1996). However, many proteins have been shown to be autoacylated, or to spontaneously bind an acyl group in a concentration-dependent manner. These proteins include actin (Bano et al. 1998), the G protein α subunit (Duncan et al. 1996), tubulin (Wolff et al. 2000), rhodopsin (Veit et al. 1998), SNAP-25 (Veit et al. 2000), and many others. Further investigation is necessary to determine if CrAT might be regulated in this way.

Data herein demonstrate that the palmitoyl-CoA-mediated CrAT inhibition was a non-competitive or mixed-model interaction, in agreement with findings in bovine spermatozoa (Huckle et al. 1983). This finding is reminiscent of the interaction between CPT1 and its inhibitor, malonyl-CoA. High carnitine appears to compete with this inhibition (Lopez-Vinas et al. 2007), defending enzyme activity. Because CPT1 and CrAT share similar carnitine binding motifs (Jogl et al. 2004), these data support the notion that carnitine acyltransferases are susceptible to heightened inhibition when local carnitine concentrations fall. This observation is particularly relevant to obesity, diabetes and other conditions of carnitine insufficiency (Noland et al. 2009, De Palo et al. 1981, Soltesz et al. 1983, Winter et al. 1989, Pregant et al. 1991, Tamamogullari et al. 1999, Poorabbas et al. 2007, Okudaira et al. 2001, Longo et al. 2006, Tein et al. 2003). Moreover, the finding that lipid-induced accumulation of long chain acylcarnitines was accompanied by a decrease in free carnitine and short chain acylcarnitine species suggests the possibility that the mitochondrial carnitine pool may be more prone to depletion than other compartments. Though regulation of carnitine between intra- and extramitochondrial compartments is poorly understood, CPT1 may gain first access to free carnitine because of its location on the outer mitochondrial membrane. Persistent production and efflux of LCACs could compromise the intramitochondrial carnitine pool. Therefore, LCAC may

sequester free carnitine away from the CrAT reaction without limiting CPT1 activity or fat oxidation.

Importantly, evidence suggests that reduced CrAT activity might impact energy metabolism at a physiological level. Recent studies determined that muscle-specific CrAT deficiency disrupts whole body glucose homeostasis (Muio et al. 2012). The glucose intolerant phenotype of CrAT^{M-/-} mice was coupled with impairments in the fasted to fed transition and a marked preference for fat oxidation. The mechanism linking CrAT to glucose oxidation could be due in part to CrAT production of acetylcarnitine, which is thought to relieve the acetyl-CoA-mediated inhibition of pyruvate dehydrogenase (PDH). Acetyl-CoA has been shown to stimulate pyruvate dehydrogenase kinases (PDKs), and to directly bind to the PDH complex, thereby diminishing its activity (Sugden and Holness 2003). In support of this model, muscle-specific deletion of CrAT resulted in complete loss of carnitine-stimulated PDH activity (Muio et al. 2012). Thus persistent inhibition of CrAT would be predicted to lower PDH activity, thereby inhibiting glucose disposal, both of which are commonly observed in obese rodents and humans (Randle et al. 1994, Sugden et al. 1995, Putman et al. 1993, Mondon et al. 1992). Further supporting the physiological role of CrAT deficiency in energy metabolism are the findings that carnitine supplementation improved insulin sensitivity and whole body glucose oxidation (Noland et al. 2009, Powers et al. 2007). These positive effects on glucose metabolism were

associated with robust increases in circulating and urinary acetylcarnitine concentrations coupled with elevated PDH activity. Moreover, adenovirus-mediated overexpression of CrAT in primary human skeletal muscle myocytes increased both acetylcarnitine efflux and glucose uptake (Muoio et al. 2012, Noland et al. 2009). Taken together, these data support the idea that deficiencies in CrAT activity exacerbate obesity-related metabolic derangements.

Lastly, we consider the biological advantage of diminished CrAT activity when long chain acyl-CoA concentrations are high. Because recent studies have demonstrated that CrAT facilitates PDH activity and glucose oxidation (Muoio et al. 2012), we hypothesize this model might contribute to the glucose-fatty acid cycle described by Randle and colleagues, 1963. These studies propose that lipid-derived acetyl-CoA inhibits PDH activity and glucose oxidation (Randle et al. 1963). CrAT opposes the Randle cycle by lowering acetyl-CoA, thereby increasing flux through PDH and glucose metabolism (Muoio et al. 2012). Therefore, CrAT-mediated acetylcarnitine generation promotes the fasted-to-fed transition. We propose that lipid-induced antagonism of CrAT might serve to counter-regulate glucose oxidation during circumstances of lipid surplus (Figure 14). Whereas this mode of regulation could provide a survival advantage during starvation, we suggest that overnutrition may cause perpetual inhibition of CrAT activity, leading to depressed PDH activity and glucose homeostasis. A better

understanding of the pathophysiological implications of reduced CrAT activity awaits future investigation.

4 Carnitine Acetyltransferase Offsets Energy Stress and Delays Muscle Fatigue During Strenuous Exercise

4.1 Introduction

Habitual exercise not only improves physical fitness and muscle strength, but also promotes metabolic health and mitigates a wide-range of medical conditions (Gordon et al. 2009, Hafer-Macko et al. 2008, Hoehner et al. 2011, Ingram and Visovsky 2007, Young 1995). Importantly however, aging and other chronic disorders are often accompanied by exercise intolerance, leading to a vicious cycle of inactivity and accelerated cardiometabolic decay. In light of an aging population and the growing worldwide prevalence of metabolic disease, efforts to understand and modify exercise fatigue have become increasingly relevant to global health.

At the most fundamental level, exercise tolerance depends on the ability of working muscles to resynthesize ATP at a pace that matches the costs of contractile activity. Sustained regeneration of ATP is driven principally by mitochondrial oxidative phosphorylation (OXPHOS), a less powerful but more efficient and higher capacity system than glycolysis. Whereas muscle mitochondrial content and respiratory capacity are well recognized determinants of exercise tolerance, several studies have shown that performance during both short and long term activities can also be influenced by the degree to which rates

of OXPHOS lag during the first several minutes of exercise and/or during stepwise increments in workload (Bangsbo et al. 2000, Howlett et al. 1998, Richardson et al. 1999, Tschakovsky et al. 1999). This phenomenon of “oxygen deficit” is measured as a transient mismatch between increased energy expenditure and estimated rates of OXPHOS assessed by whole body VO_2 kinetics. The debt incurred during these transitions is paid in large part by anaerobic glycolysis at the expense of lactate accumulation and accelerated glycogen depletion, both of which weigh negatively on muscle energy economy and performance.

Rapid adjustments in the rate of OXPHOS depend on availability of oxygen as the final electron acceptor and a steady supply of electron donors in the form of NADH and FADH_2 . Two general theories have been proposed to account for the foregoing lag in oxygen consumption during abrupt increases in exercise intensity. The first suggests a limitation at the level of oxygen delivery to electron transport chain (ETC), whereas an alternative view proposes deficits in acetyl group availability, also referred to as metabolic inertia. Acetyl-CoA holds a prominent position in exercise bioenergetics as the two carbon intermediate of glucose, fatty acid and amino acid catabolism. Because acetyl-CoA fuels the tricarboxylic acid (TCA) cycle, the main source of the reducing equivalents that drive OXPHOS, a deficit in acetyl-CoA supply would be predicted to limit the rate of oxidative ATP production.

Although limitations in oxygen and carbon supply are widely viewed as co-contributors to oxygen deficit (Greenhaff et al. 2002, Knight et al. 1993, Richardson et al. 1995), the metabolic inertia theory draws attention to nutritional maneuvers that might augment the provision of specific acetyl group precursors. Relevant to this idea is emergent evidence that mitochondrial acetyl-CoA balance can be nutritionally regulated via the carnitine-dependent enzyme, carnitine acetyltransferase (CrAT) (Muoio et al. 2012). This enzyme, which is highly enriched in muscle and heart (Noland et al. 2009), converts acetyl-CoA and other short chain acyl-CoAs to their membrane permeant acylcarnitine counterparts, thereby permitting mitochondrial and tissue efflux of excess carbons. Dietary supplementation with L-carnitine promotes net export of acetylcarnitine, which is accompanied by enhanced glucose tolerance when administered to obese rodents and humans (Noland et al. 2009). These findings raised the question of why muscle tissues express such high amounts of an enzyme that essentially siphons acetyl-CoA from the TCA cycle. The answer probably relates to the freely reversible nature of CrAT, which interconverts acetyl-CoA and acetylcarnitine with an equilibrium constant of ~ 1.5 (Farrell et al. 1984, Pieklik et al. 1975). Thus, in theory, CrAT acts to buffer the mitochondrial pool of acyl-CoA, which implies an important role for this enzyme in offsetting acetyl group deficit during transitions from low to high energy demand. Although a number of studies have reported that both intramuscular and circulating levels of

acetylcarnitine increase during exercise (Sahlin et al. 1990, Hiatt et al. 1989, Putman et al. 1993, Watt et al. 2002), suggesting elevated flux through the CrAT reaction, the physiological relevance of these observations have remained uncertain. Herein, we examined the impact of skeletal muscle-specific CrAT deficiency on muscle bioenergetics, acetyl group balance and exercise performance. Our findings not only establish an important role for CrAT in regulating muscle energy economy during contraction, but also suggest that nutritional and/or pharmacological strategies aimed at promoting CrAT activity could prove useful for delaying muscle fatigue and combatting exercise intolerance.

4.2 Materials and Methods:

Animals: Animal studies were approved by the Duke University Institutional Animal Care and Use Committee. Mice were group housed and allowed *ad libitum* access to water and standard chow (Purina Rodent Chow no. 5015, Purina Mills, St. Louis, MO, USA). Mice were randomly selected to receive two weeks of supplementation with pH-neutralized drinking water containing 0 or 1 mg/ml L-carnitine. Mice were anesthetized using an intraperitoneal injection of Nembutal with the dose of 100 mg/kg body weight. Tissue samples were frozen in liquid nitrogen and stored at -80°C. Tissues were ground into powder and processed in CellLytic buffer (Sigma Chemicals, St. Louis, MO) by freeze

fracturing three times and sonication at five times one second pulses on setting five.

Mitochondrial Isolation: Skeletal muscle mitochondria were prepared according to (Noland et al. 2009).

Carnitine acetyltransferase activity: CrAT activity was assessed according to (Muoio et al. 2012) and (Seiler et al. 2013).

Western blots: Protein was isolated using CellLytic lysis buffer (Sigma Chemicals, St. Louis, MO). A bicinchoninic acid (BCA) kit (Sigma Chemicals, St. Louis, MO) was used to quantify protein. Protein (50µg from cell/tissue lysates) was separated by SDS-PAGE, transferred to nitrocellulose, and incubated with antibodies prepared with fish gelatin blocking buffer (0.5% fish gelatin, 2% 10xPBS, 1 mg/ml casein and 15 mM NaN₃). IgG-conjugated AlexFluor fluorescent secondary antibodies (Invitrogen) were used with the Li-COR (Li-COR Biosciences) to visualize bands. Protein expression was normalized to total protein as determined by MemCode staining (Thermo Scientific). The primary CrAT antibody was a generous gift from the laboratory of Dr. Fausto Hegardt.

Metabolite Analysis: Tissue and plasma samples were processed and analysed by the Sarah W. Stedman Nutrition and Metabolism metabolomics/biomarker core laboratory. Acylcarnitine measurements were made using flow injection tandem mass spectrometry and sample preparation methods described in (An et al. 2004) and (Haqq et al. 2005). Acyl-CoA esters were analyzed using a method based on a previously published report (Magnes et al. 2005) which relies on the extraction procedure described by (Deutsch et al. 1994). The CoAs were further purified by solid phase extraction as described by (Minkler et al. 2008). Nucleotides were measured based on a previously reported method (Cordell et al. 2008). Chromatographic separations were performed using an Agilent Technologies (Santa Clara, CA) 1200 HPLC system and a Chromolith FastGradient RP-18e 50-2mm column (EMD Millipore, Billerica, MA, USA).

Phosphocreatine Content: PCr and creatine analysis was done according to (Hu 1975) and (Harris et al. 1974).

Glycogen Content: Glycogen was assessed using approximately 20 mg of powdered skeletal muscle. 0.5 ml of 1 N HCl was added to the tissue in a 2.0-ml screw top tube and homogenized at 25K RPM for 30 seconds. The samples were then boiled for 90 minutes. The tubes were cooled to room temperature and 0.5

ml of 1 N NaOH was added. Glycogen content was assessed in the samples at 340nm after addition of glucose assay reagent (Sigma).

Lactate: Lactate was assessed at 340 nm. Lactate assay buffer consisted of 175 mM hydrazide sulfate, 68 mM glycine, 2.9 mM EDTA, and 11.3 mM NAD⁺, pH 9.5. Lactate standard (Sigma) was made up at 3, 1.5, 0.75, 0.375 and 0.1875 mM. 20uL sample or standard were added with 200 uL assay buffer. Background absorbance was measured. 10 uL LDH enzyme (Sigma), made up at 500 units/ml in water, was then added rapidly and absorbance was monitored for 60 minutes. Upon kinetic plateau, final absorbance was taken. Lactate was determined according to the standard curve.

Exercise Studies: Because of the inherent subjectivity of treadmill running to exhaustion, exercise studies were carried out in a blind fashion. Before exercise testing, three month old mice were acclimated to the treadmill for three days prior to the study. Acclimation consisted of a three minute run with one minute at each of the first three speeds in the respective protocol being tested. Exhaustion was defined as remaining on the shock grid for more than 10 seconds with nudging.

Endurance: Endurance exercise was analyzed at a fixed slope of 10°. Mice began running at 8 m/min. Treadmill speed was increased 2 m/min every 15

minutes until 16 m/min was attained. At this point, mice were held at a consistent speed until exhaustion.

Endurance with High Intensity Ramping: The transition from endurance to high intensity running was completed with a fixed slope of 10°. Similar to the endurance protocol described above, mice began running at 8 m/min and increased 2 m/min every 15 minutes for 1 hour total. After the initial hour, the mice were transitioned into a higher intensity exercise with 1 minute at 20 m/min, then 10 minutes at 23 m/min, with an increase of 3.5 m/min every 10 min until exhaustion. Data presented represent the ramping up period only.

High Intensity: Exercise capacity during high intensity running was determined using an enclosed treadmill (Columbus instruments), attached to the Comprehensive Laboratory Animal Monitoring System (CLAMS) with a fixed incline of 10°. Mice began running at 14 m/min and increasing speed 3 m/min every 3 minutes until exhaustion. Measurements were collected every 30 seconds during which air flow was set to 0.6 l/min.

Ex-vivo muscle Stimulation and Acetylcarnitine Oxidation: Braided silk suture loops were attached to muscle tendons and excised from the mouse. Muscles were placed in pre-warmed KHB buffer (pH 7.4) which contained (in mmol/l) 118 NaCl, 4.7 KCl, 2.52 CaCl₂, 1.64 MgSO₄, 24.88 NaCO₃, 1.18 KH₂PO₄, 5.55 glucose and 2.0 Na-pyruvate. After placement in a Radnoti 2 mL tissue bath

system, muscles were tensed to 1 gram and allowed to relax for 15 minutes. Muscles were then re-tensed to 1 gram and allowed to rest 5 minutes. This re-tense/5 minute rest was repeated three times before initiation of stimulation. KHB buffer was used in muscle transport and tensing, while low glucose KHB (+2mM glucose, no pyruvate) was used during stimulation. The following stimulation protocol was used unless otherwise stated: stimulation rate: 60 pps, delay: 2000 ms, duration: 300 ms, volts: 20 per chamber. O₂ flow and temperature (25°C) were kept constant throughout stretching and stimulation. Acetylcarnitine (Sigma), was made up at 1 M in H₂O and added at a final concentration of 5 mM to stimulating muscles 30 seconds after initiation of contraction. This allowed for normalization of contracting muscles prior to acetylcarnitine addition. Acetylcarnitine oxidation was assessed by capturing media ¹⁴CO₂ from 200 μM [1-¹⁴C]acetylcarnitine (ARC 1615) during rest or stimulation. Oxidation studies were done in low glucose KHB buffer with 3 mg/mL HEPES added.

Statistics: JMP software version 7.0 (SAS Institute, Cary, NC) was used to perform multivariate correlation analyses of metabolites. Other statistical analyses were performed using SigmaStat (SysStat Software, Inc., Point Richmond, CA) or the Microsoft Excel statistical package. Within-group responses to experimental manipulations were evaluated using a paired *t* test,

where appropriate. All data are presented as mean S.E., and the level of significance was established *a priori* at *p* less than or equal to 0.05.

4.3 Results

Skeletal Muscle is a Primary Site of CrAT Activity. To characterize the relationship between CrAT and muscle function we first examined the fiber type distribution of the enzyme as well as its regulation in response to exercise. In general, CrAT protein abundance was highest in the most oxidative/red muscles as compared to those comprised of a more glycolytic/white fiber types (Figure 15A). This expression pattern was only partly attributable to total mitochondrial content, as CrAT abundance was also dissimilar among isolated mitochondria preparations harvested from different muscles (Figure 15B). Interestingly, mitochondrial abundance of CrAT tracked most closely with muscle enrichment of type IIa (intermediate) rather than type I (purely oxidative) myofibers. Skeletal muscle mRNA levels of CrAT increased 4-fold in response to an acute 90 minute mid-intensity exercise bout (Figure 15C). This was evident immediately after and up to 24 h after exercise as compared to the rested controls. Likewise, genetically-engineered overexpression of the exercise-inducible transcriptional co-activator, PGC1 α , increased *crat* gene expression in muscles from MCK-PGC1 α transgenic mice (Figure 15D) and in primary human skeletal myocytes

grown in culture (Figure 15E). In aggregate, these results point to a role for CrAT in meeting the energetic needs of exercising muscles.

To further explore the functional relevance of CrAT, we used the Lox-P/Cre system described in Experimental Procedures to generate mice lacking *crat* specifically in skeletal muscle (CrAT^{SM-/-}). This strategy resulted in 80-90% loss of CrAT activity in skeletal muscles without affecting that in heart (Figure 15F-G). Interestingly, muscle quantities of acetylcarnitine, the principal product of CrAT, were only 27% lower in CrAT^{SM-/-} as compared to control mice (Figure 15H), indicating that other tissues contribute to the skeletal muscle pool of this metabolite. As predicted, CrAT deficiency was accompanied by a 1.5 fold increase in muscle concentrations of acetyl-CoA (Figure 15H).

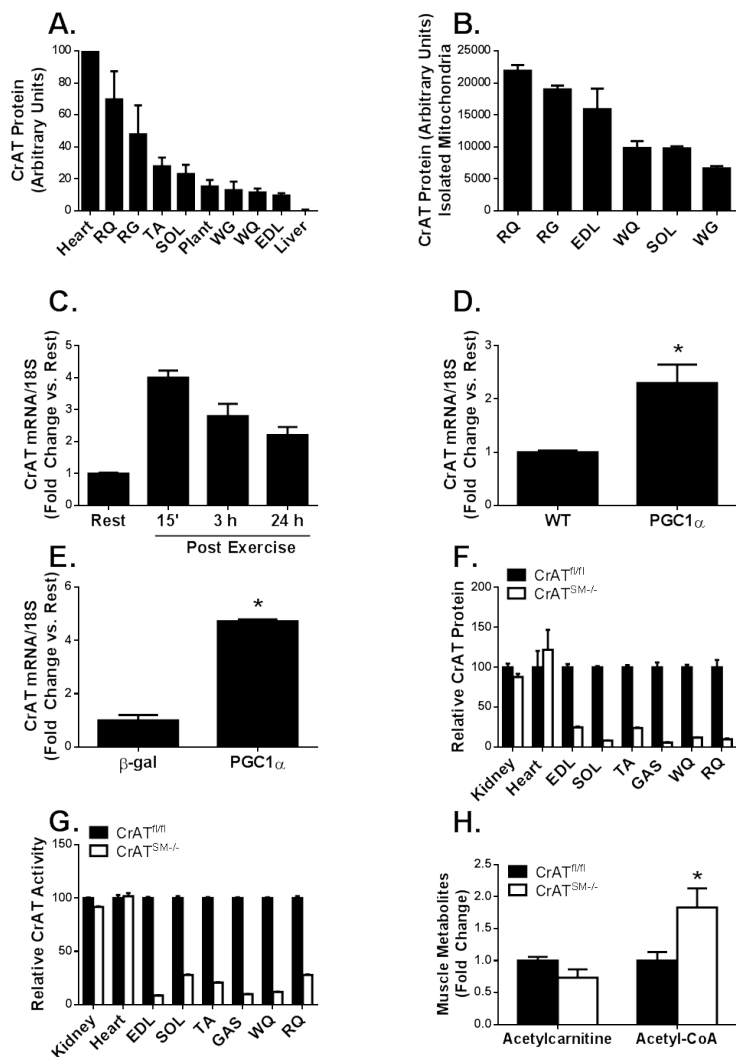


Figure 15: Tissue-Specific Targeting of CrAT Activity

CrAT protein abundance was determined in three month old rat heart, red quad (RQ), red gastroc (RG), tibialis anterior (TA), soleus (SOL), planaris (Plant), white gastroc (WG), white quad (WQ), EDL and liver tissue homogenates (A) and isolated mitochondria (B). CrAT mRNA expression normalized to 18S was determined (C) in mouse gastrocnemius muscle at rest and 15 minutes, 3 hours and 24 hours after a 90 minute exercise bout (D) in gastrocnemius homogenates from MCK-PGC1 α transgenic and wild type (WT) control mice and (E) in primary human skeletal myocytes treated with recombinant adenovirus encoding β -galactosidase or rat PGC1 α . Tissue-specific CrAT deletion was confirmed by analyzing CrAT protein (F) and enzyme activity (G) in multiple tissues (n = 3-6/group). Acetylcarnitine and acetyl-CoA in quadriceps muscles were measured by mass spectrometry (H; n = 6/group). * Genotype effect (p<0.05).

Skeletal muscle-specific loss of CrAT compromised exercise performance despite increased fat oxidation. To test the impact of CrAT deficiency on exercise performance, CrAT^{SM-/-} and littermate controls were subjected to two different treadmill protocols (Figure 16A), both designed to challenge the capacity of the muscle to cope with several abrupt increments in energy demand. The first was an endurance protocol with high intensity ramping during the latter half of the run. Time and distance to exhaustion were decreased 18% and 29%, respectively in CrAT^{SM-/-} compared CrAT^{fl/fl} mice (Figure 16B). During a second, higher intensity test the frequency and magnitude of the increments were increased and whole body respiration was monitored by indirect calorimetry. Again, the CrAT^{SM-/-} mice performed poorly and completed only 66% of the distance covered by the control group (Figure 16C). CrAT^{SM-/-} mice tended to have higher O₂ consumption and lower CO₂ production throughout the exercise, resulting in a lower average RER of 0.931 compared to 0.992 in the CrAT^{fl/fl} controls (Figure 16D-F).

Because a reduction in RER typically indicates a substrate shift in favor of fat oxidation, we proceeded to examine potential changes in β -oxidation potential using isolated muscle mitochondria. Consistent with the whole body measures, maximal rates of palmitoylcarnitine-supported respiration were increased 38% in mitochondria harvested from CrAT deficient versus control muscles (Figure 16G). This was accompanied by 1.5-2.0-fold increases in muscle mRNA expression of

several β -oxidation enzymes, including CACT, CPT2, VLCAD and MCAD (Figure 16H). We suspect that this molecular remodeling of the β -oxidation machinery might be at least partly due to elevated levels of long chain acyl-CoAs (Muoio et al. 2012) and consequent activation of lipid-responsive transcription factors such as PPAR α . Collectively, these results revealed an atypical physiologic phenotype wherein enhanced capacity for fat oxidation was accompanied by reduced exercise tolerance.

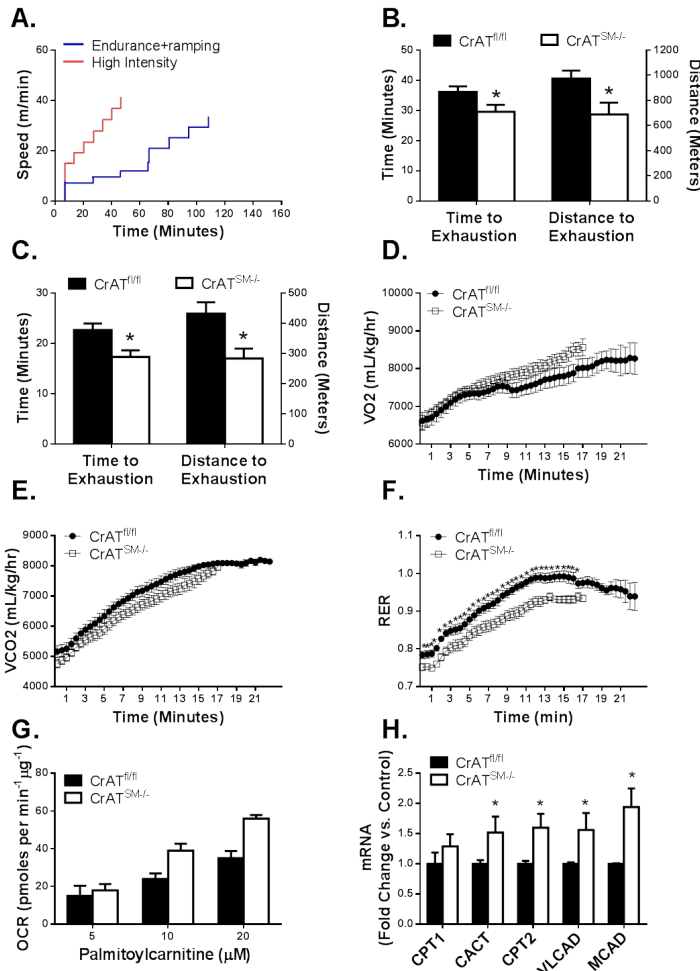


Figure 16 CrAT Deficiency Compromises Exercise Performance Despite Increased Fat Oxidation

12 week old mixed gender mice were run to exhaustion on two treadmill protocols described (A). Time and distance to exhaustion were determined during (B; n = 10/group) endurance exercise with high intensity ramping (data shown represent the ramping period only), and (C; n = 10/group) high intensity exercise. Indirect calorimetry was performed using an enclosed metabolic treadmill (Columbus Instruments) and VO_2 (D), VCO_2 (E), and respiratory exchange ratio (RER) were calculated (F; n = 10/group). State 3 mitochondrial respiration was determined in $CrAT^{fl/fl}$ and $CrAT^{SM-/-}$ gastrocnemius muscle using the Seahorse (Seahorse Bioscience) after the addition of 5 mM malate, 1 mM carnitine and 5, 10 or 20 mM palmitoylcarnitine (G; n = 5/group). mRNA expression normalized to 18S was determined for carnitine palmitoyltransferase 1 (CPT1), carnitine acylcarnitine translocase (CACT), carnitine palmitoyltransferase 2 (CPT2), very long chain acyl-CoA dehydrogenase (VLCAD) and medium chain acyl-CoA dehydrogenase (MCAD) in $CrAT^{fl/fl}$ and $CrAT^{SM-/-}$ mouse gastrocnemius muscle (H; n = 6/group). * Genotype effect ($p < 0.05$).

CrAT deficiency disrupted acetyl-CoA buffering capacity and exercise bioenergetics. To gain insight into the biochemical underpinnings of premature fatigue in CrAT^{SM-/-} mice, we evaluated muscle metabolite levels prior to exhaustion, using tissues collected from a second set of mice that were subjected to only 12 min of the high intensity treadmill running. Metabolites were measured in muscles harvested 10 min or 1 h after exercise cessation and compared against those from rested controls. In CrAT^{fl/fl} control mice, muscle concentrations of both acetylcarnitine (Figure 17A) and acetyl-CoA (Figure 17B) were remarkably stable at these time points. By contrast, acetylcarnitine/acetyl-CoA balance fluctuated dramatically in muscles from the CrAT^{SM-/-} mice (Figure 17C). Acetylcarnitine content in muscles from CrAT^{SM-/-} trended lower in the rested state (Figure 17A), but then increased to levels comparable to the control group during the post-exercise period. Thus, exercise appeared to promote acetylcarnitine uptake from the circulation into working muscles. Conversely, acetyl-CoA concentrations in muscles from CrAT^{SM-/-} vs. CrAT^{fl/fl} mice were elevated 2-fold at rest but then fell dramatically in response to exercise, implying a connection between acetyl group deficit and early fatigue. In the CrAT^{SM-/-} mice, acetyl-CoA levels were partly restored during the 1 h recovery.

Abrupt fluctuations in muscle energy demand are immediately buffered by PCr, which donates its high energy phosphoryl group to ADP in a freely

reversible phosphotransfer reaction catalyzed by creatine kinase (CK). Muscle contraction favors CK flux in the forward direction to generate ATP and Cr, whereas the opposite holds true during exercise recovery. Because the limited myocellular storage pools of PCr can provide for only a few seconds of maximal activity, continuous regeneration of PCr is critical to muscle performance and recovery. Within 10 minutes of exercise cessation, the PCr pool in muscles from control mice was not only fully replenished but actually elevated compared to the rested state (Figure 17D). This post-exercise overshoot is typical of a short duration, high intensity activity and indicative of robust cardiometabolic fitness (Korzeniewski et al. 2003, Korzeniewski et al. 2005, Sahlin et al. 1997, Meyer et al. 1994). Notably, CrAT deficient muscles lost capacity to quickly stockpile PCr during the same recovery period (Figure 17D).

Deficiencies in oxidative ATP production increase reliance on glycolysis and thus muscle glycogen reserves. Interestingly, in the rested state muscle glycogen levels were elevated in CrAT^{SM-/-} mice, perhaps due to the foregoing shift in substrate selection (Figure 15E). However, despite elevated fat oxidation during the treadmill test (Figure 16E), exercise-induced depletion of muscle glycogen was 3.5-fold greater in the CrAT^{SM-/-} mice (Figure 17F). Taken together, these findings provide strong support for the acetyl group deficit theory of muscle fatigue and offer the first conclusive evidence that CrAT is essential for

optimizing acetyl-CoA balance and muscle energy economy during and after exercise.

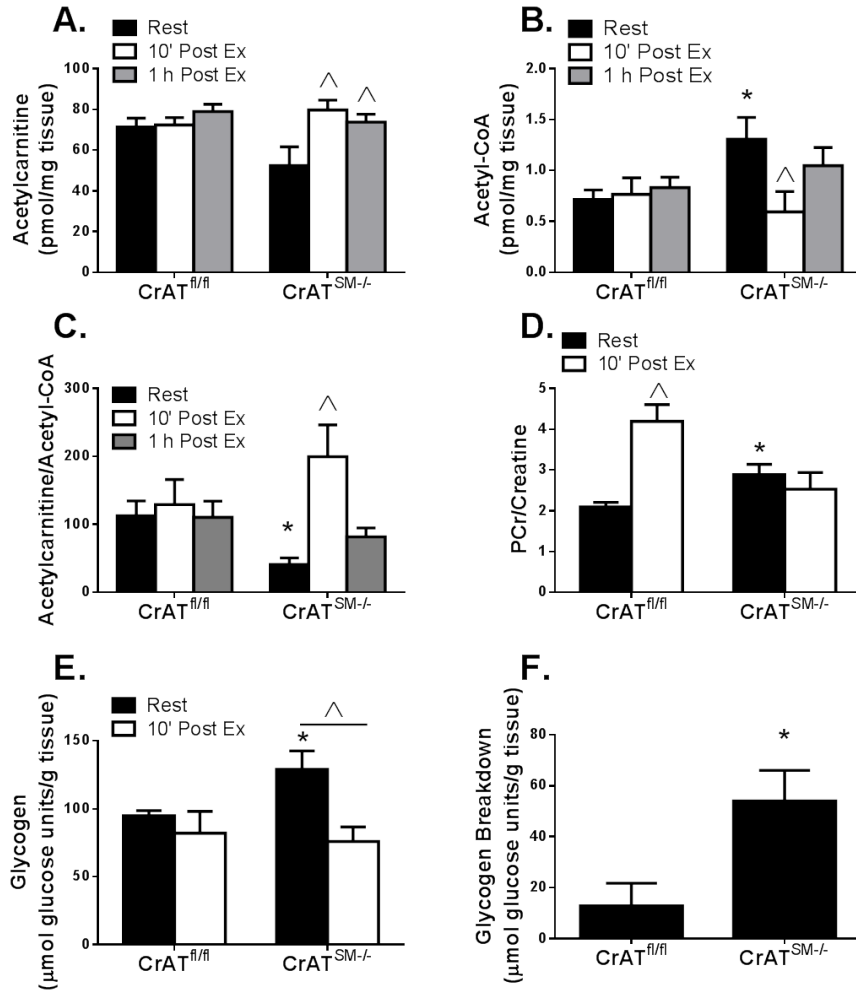


Figure 17 Loss of Acetyl-CoA Buffering Capacity and Exercise Bioenergetics in CrAT^{SM-/-} Mice

Quadriceps muscle homogenates were harvested at rest, 10 minutes and one hour after a 12 minute high intensity exercise. Tandem mass spectrometry was used to assess acetylcarnitine (A), acetyl-CoA (B), and the acetylcarnitine:acetyl-CoA ratio (C; n = 6/group). Phosphocreatine (PCr) (D), glycogen (E) and the exercise-induced glycogen depletion, calculated as % decreased from rest (F) were also determined (n = 5-8/group). * Genotype effect (p<0.05), ^ Exercise effect (p<0.05).

Exogenously supplied acetylcarnitine delayed fatigue and improved energy economy in a CrAT- dependent manner. Results of the exercise tolerance studies suggested that CrAT-derived acetylcarnitine supports mitochondrial respiration when supply of acetyl-CoA from other fuel sources is limiting. This hypothesis was subsequently confirmed in isolated mitochondria, which maintained high rates of acetylcarnitine-supported respiration in a CrAT-dependent manner (Figure 18A). Also noteworthy, acetylcarnitine permitted higher rates of respiration than palmitoylcarnitine, regardless of whether the two substrates we evaluated separately (Figure 18B) or in combination (Figure 18C).

Results presented in Figure 17A suggested that exercise stimulates muscle acetylcarnitine uptake. To test this possibility we used a muscle incubation bath system to examine contraction-induced changes in the oxidation rates of exogenously supplied [^{14}C]acetylcarnitine in isolated soleus and EDL. As predicted, electrical stimulation applied during a 10 min incubation caused a marked 10-fold increase in acetylcarnitine catabolism to [^{14}C]CO₂ as compared to rested/unstimulated EDL muscles (Figure 18D). This outcome was fully dependent on CrAT activity. Moreover, in control muscles, provision of 5 mM acetylcarnitine enhanced force generation and delayed fatigue, such that time to half maximal force generation increased from 70 to 106 seconds (Figure 18E).

By contrast, acetylcarnitine had no effect on the force-fatigue properties of EDL from CrAT^{SM-/-} mice (Figure 18F).

We then repeated these experiments and assessed muscle energy charge after four minutes of contraction, prior to fatigue. As expected, pilot experiments performed with control mice confirmed robust contraction-induced shifts in muscle content of high energy phosphagens (Table 3). In control but not CrAT-deficient muscles, exogenously supplied acetylcarnitine defended the muscle PCr pool, resulting in a near two-fold increase in the post-contraction PCr/Cr ratio (Figure 18G). In addition to the CK reaction, adenylate kinase also plays a key role in exercise bioenergetics by catalyzing phosphoryl group transfer from one ADP to another, thereby regenerating one ATP along with AMP. The latter molecule can then be deaminated to produce IMP as a critical step in the adenine nucleotide cycle. When acetylcarnitine was provided to control muscles, post-contraction levels of the foregoing adenine nucleotides reflected a favorable shift in energy charge. Thus, acetylcarnitine lowered both the AMP/ATP and IMP/ATP ratios, indicative of improved metabolic efficiency. In support of this observation, lactate efflux declined after the addition of exogenous acetylcarnitine. By contrast, these same indices of energy stress were completely unresponsive to the acetylcarnitine treatment in muscles from the CrAT^{SM-/-} mice (Figure 18H-J).

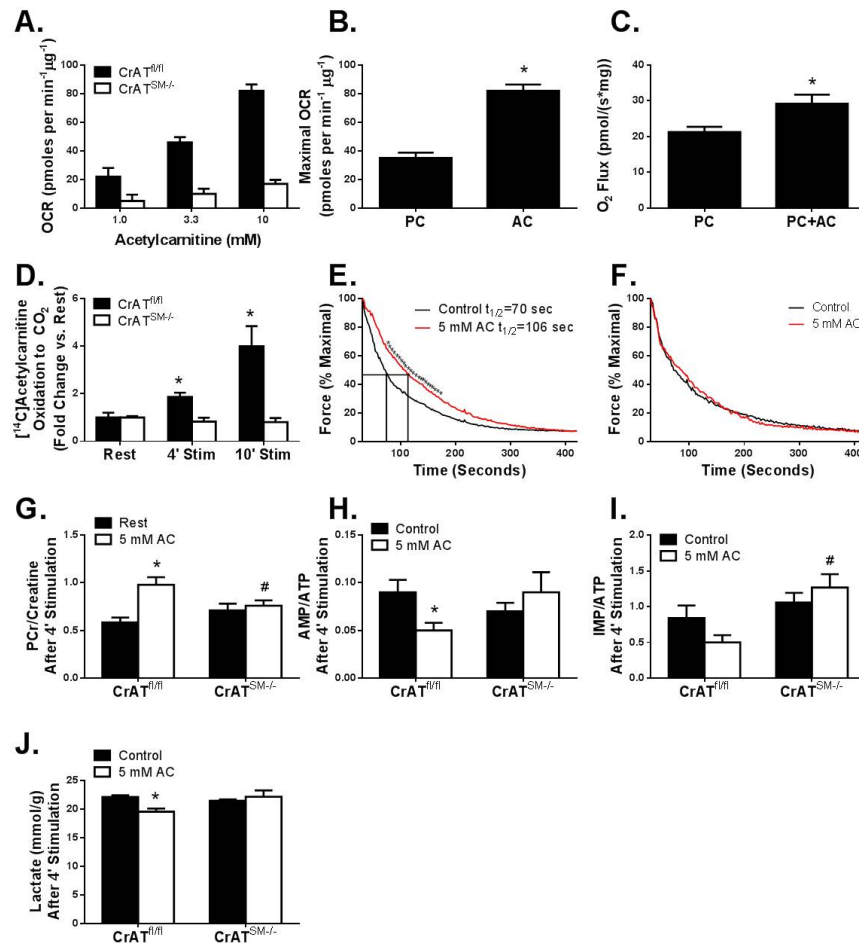


Figure 18 Exogenously Supplied Acetylcarnitine Delayed Fatigue and Improved Energy Economy in a CrAT- Dependent Manner

Seahorse measurements during state 3 respiration were conducted in gastrocnemius mitochondria (A; n = 5/group) after the addition of 1 mM carnitine and 1, 3.3 or 10 mM acetylcarnitine in CrAT^{fl/fl} and CrAT^{SM-/-} mice and (B; n = 5/group) at maximal palmitoylcarnitine (PC) and acetylcarnitine (AC) respiration in CrAT^{fl/fl} mice, determined to be 20 μM PC and 10 mM AC. The OROBOROS system was employed to determine state 3 respiration of permeabilized plantaris fiber bundles at maximal (100 μM) palmitoylcarnitine respiration ± 5 mM acetylcarnitine addition (C; n = 8/group). A Radnoti tissue bath system was used to determine [¹⁴C]Acetyl carnitine oxidation to CO₂ in CrAT^{fl/fl} and CrAT^{SM-/-} EDL after a 4 and 10 minute stimulation (D; n = 4/group). Force generation and time to fatigue were determined ± 5 mM acetylcarnitine addition in CrAT^{fl/fl} (E) and CrAT^{SM-/-} (F) littermates (n = 6/group). EDL were flash frozen after a 4 minute stimulation ± 5mM acetylcarnitine and (G) the PCr/Creatine ratio, (H) AMP/ATP, (I) IMP/ATP and (J) media lactate were determined. * acetylcarnitine effect (p<0.05), # genotype effect (p<0.05).

Table 3: Contraction-Induced Changes in Muscle Content of High Energy Phosphagens

EDL samples were harvested from control mice and flash frozen immediately after resting control or four minute stimulation. PCr and Cr content were analyzed and the PCr/Cr ratio was calculated. Liquid chromatography-Mass spectrometry was used to assess ATP, ADP, AMP, IMP and the ratios ADP/ATP, AMP/ATP and IMP/ATP. Data are expressed as $\mu\text{mol/g}$ tissue and represent means \pm S.E. from 5-8 muscles per group. * $p < 0.05$, rest vs. stimulated.

	Rested	Stimulated
PCr	21.3 \pm 1.9	12.4 \pm 1.2 *
Cr	10.6 \pm 0.6	16.7 \pm 0.7 *
ATP	1.6 \pm 0.1	1.0 \pm 0.2 *
ADP	0.1 \pm 0.01	0.9 \pm 0.04 *
AMP	0.05 \pm 0.009	0.09 \pm 0.02 *
IMP	0.47 \pm 0.07	10.8 \pm 0.09 *
PCr/Cr	2.2 \pm 0.3	0.7 \pm 0.09 *
ADP/ATP	0.07 \pm 0.005	1.0 \pm 0.14 *
AMP/ATP	0.03 \pm 0.007	0.09 \pm 0.01 *
IMP/ATP	0.3 \pm 0.06	0.8 \pm 0.2 *

Carnitine supplementation enhanced exercise performance in control but not CrAT^{SM-/-} mice. To determine whether the benefits of enhanced acetyl-group buffering capacity would take effect at a whole body level, we performed the same two graded treadmill tests described in Figure 16, but after two and four weeks of dietary L-carnitine supplementation. Importantly, the genotype and treatment groups were blinded to the treadmill operators. Consistent with previous reports (Noland et al. 2009, Makowski et al. 2009), administration of L-carnitine in the drinking water increased circulating levels of free carnitine and acetylcarnitine by 30% and 20%, respectively, regardless of genotype (Figure 19A-B). In control mice, L-carnitine supplementation improved distance to exhaustion by 30% and 27% during the endurance with ramping and the high intensity regimens, respectively. This carnitine-mediated improvement of exercise performance was absent in the CrAT^{SM-/-} mice (Figure 19C-D). Serum levels of acetylcarnitine measured immediately after the second treadmill test were increased 34-38% in both carnitine supplemented groups (Figure 19E), while intramuscular levels of free, esterified and total carnitine levels were unchanged (Figure 19F). Thus, in line with results of the muscle incubation experiments (Figure 18E), increased supply of acetylcarnitine to working muscles was accompanied by enhanced exercise tolerance in CrAT^{fl/fl} mice but not CrAT^{SM-/-} counterparts. In aggregate, these results support the idea that nutritional strategies aimed at increasing acetyl-CoA buffering capacity have the

potential to augment energy efficiency and delay muscle fatigue during strenuous physical activity.

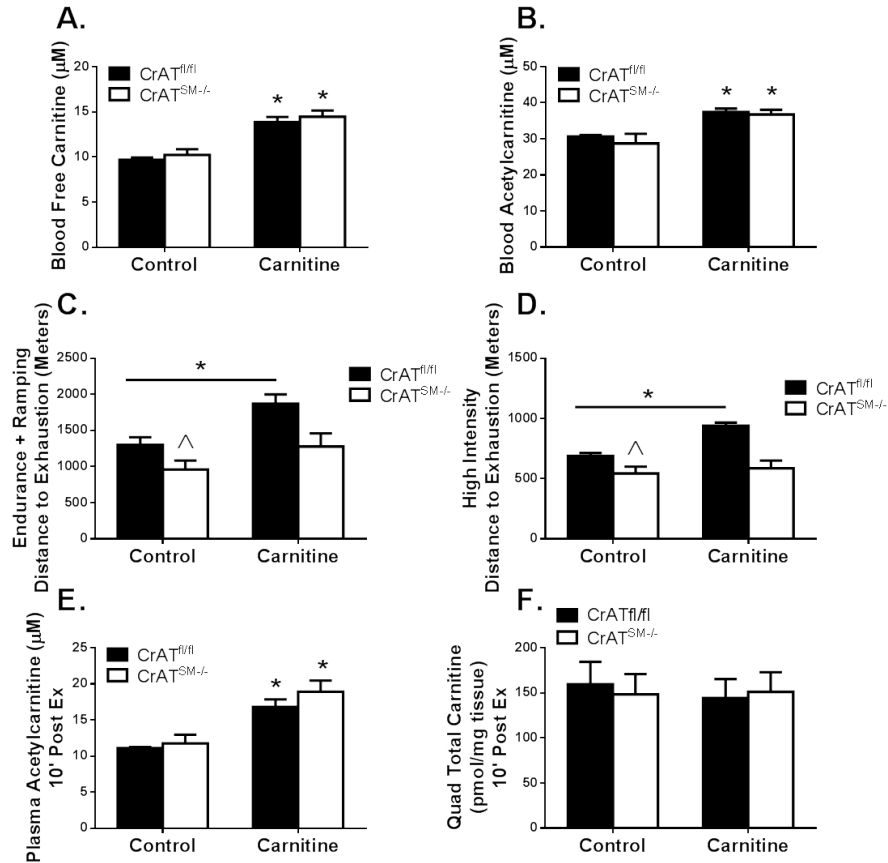


Figure 19: Acetylcarnitine Enhanced Exercise Performance in Control but Not CrAT^{SM-/-} Mice

Mice were randomly selected to receive supplementation with pH-neutralized drinking water containing 0 or 1 mg/ml l-carnitine for two and four weeks. Tandem mass spectrometry was used to assess free carnitine (A) and acetylcarnitine (B) in blood spots taken from the tail vein. Time and distance to exhaustion were determined during (C; n = 6/group) endurance exercise with high intensity ramping (data shown represent the ramping up period only), and (D; n = 6) high intensity exercise. Plasma acetylcarnitine (E) and quad total carnitine (F; free carnitine + acetylcarnitine) were determined after a 90 minute endurance with ramping bout. * p < 0.05 carnitine effect, ^ genotype effect (p < 0.05).

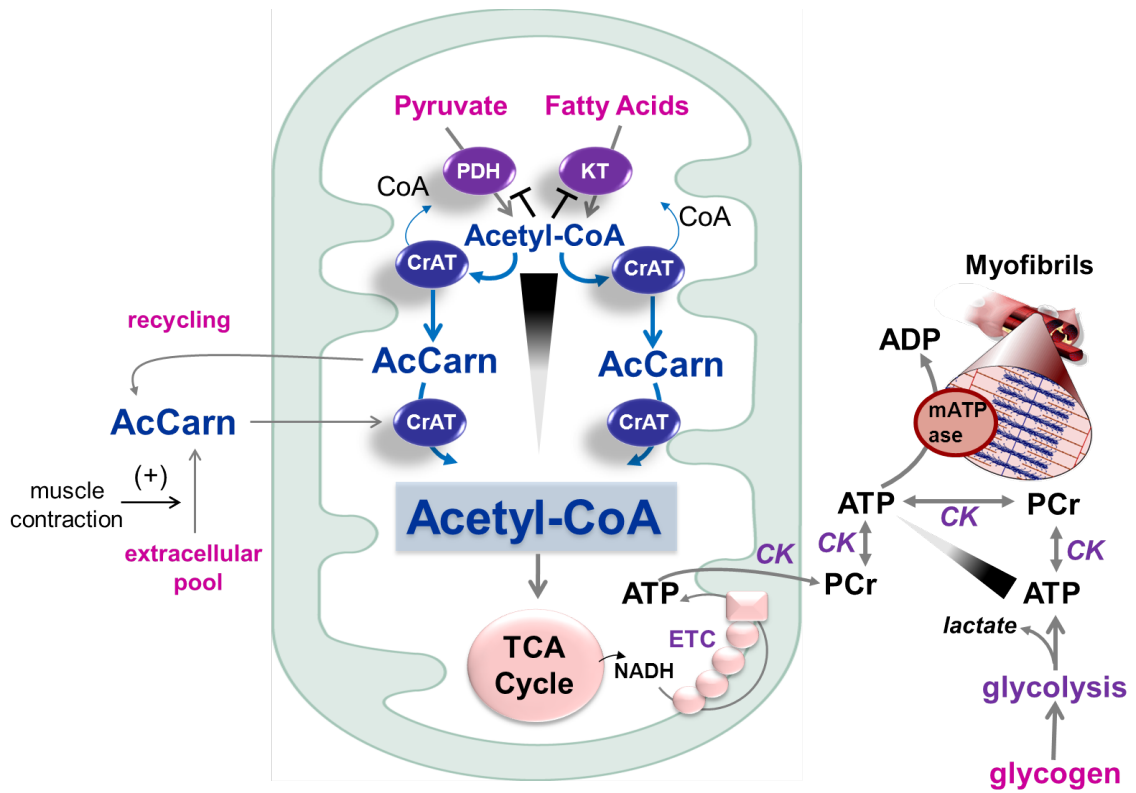


Figure 20: Proposed Role of CrAT in Mitochondrial Acetyl-CoA Buffering and Acetyl Group Transfer During Exercise

Acetyl-CoA is the crucial catabolic intermediate that fuels the tricarboxylic acid (TCA) cycle, which in turn serves as the main source of reducing equivalents (NADH) that support oxidative regeneration of ATP by the electron transport chain (ETC). During transitions from low to high exercise workloads, TCA cycle flux must increase to keep pace with the high ATP demands of muscle contraction. A shortfall in acetyl-CoA provision will force heavy reliance on substrate level phosphorylation, resulting in depletion of phosphocreatine (PCr) and muscle glycogen reserves, along with production of lactic acid and other deleterious metabolic byproducts. CrAT functions to sustain high rates of oxidative ATP regeneration via two proposed mechanisms. First, contraction-induced recycling and/or import of acetylcarnitine (AcCarn) supplies a readily available source of acetyl group donors to buffer transient deficits in rates of glucose, amino acid and fatty acid catabolism. Secondly, CrAT permits rapid and efficient delivery of acetyl groups from their site of production to the TCA cycle (blue arrows). By residing in close proximity to the various enzymatic sources of acetyl-CoA (e.g. pyruvate dehydrogenase (PDH) and ketothiolase (KT)), CrAT alleviates kinetic hindrances due to product inhibition while also regenerating essential cofactor (CoA) for continued catabolic flux. Additionally, by acting as a conduit for acetyl group transfer, CrAT overcomes the thermodynamic inefficiencies of diffusional flux, which requires an energetically unfavorable gradient profile (black triangle). This model of substrate transfer through a series of near-equilibrium reactions is conceptually analogous to the phospho-energy transfer networks driven by the adenylate kinase and creatine kinase (CK) systems.

4.4 Discussion

Previous studies have suggested that rapid adjustments in the rate of OXPHOS during transitions from low to higher workloads might be limited by a lag in enzyme activation, leading to transient deficits in metabolic flux and acetyl-CoA production. In the current investigation we hypothesized that the acetyl group buffering capacity of CrAT plays a critical role in mitigating and/or compensating for metabolic inertia. Fitting with this prediction, we provide new and compelling evidence that CrAT activity influences acetyl-CoA balance, muscle bioenergetics and time to fatigue during intense exercise. Muscle-specific deletion of CrAT compromised muscle energy economy and contractile performance, whereas enhancement of acetyl-CoA buffering capacity via dietary L-carnitine supplementation improved exercise tolerance in a CrAT-dependent manner. These findings are consistent with the acetyl group deficit theory of fatigue and suggest that strategies aimed at augmenting CrAT activity could have value as an ergogenic aid.

Recent studies found that CrAT protein is most abundant in skeletal muscle and heart, tissues specifically designed for work (Noland et al. 2009). Moreover, in the present study we found that CrAT mRNA expression increases in response to acute exercise (Figure 15C). Together, these findings point to a role for CrAT in contraction. However, heretofore, experimental evidence to support this idea was lacking. Importantly, our study fills this gap with three major

findings. First, we provide evidence that CrAT indeed has a critical role in strenuous exercise performance using a novel mouse model of CrAT skeletal muscle deficiency. Second, we used ex-vivo analysis to demonstrate that CrAT offsets the contraction-mediated acetyl-CoA deficit and maintains high rates of phosphocreatine (PCr) regeneration, thereby improving mitochondrial energetics and delaying fatigue. Lastly, we demonstrated that supplementation with carnitine, a primary substrate for the CrAT reaction, improved exercise performance in CrAT^{fl/fl} but not CrAT^{SM-/-} mice. In aggregate, these results establish a critical role for CrAT in combatting contraction-induced energy stress and fatigue.

A growing literature has suggested that acetyl-CoA might be a limiting factor for mitochondrial ATP generation during exercise. This acetyl group deficit theory was driven by studies using dichloroacetate (DCA) to elevate acetyl group availability prior to and during contraction. DCA, a chemical compound that has been shown to combat PDH phosphorylation thereby activating the enzyme complex, drives pyruvate flux through PDH. The resulting expansion of the acetylcarnitine pool resulted in improved exercise performance and mitochondrial energetics (Howlett et al. 1999, Timmons et al. 1998, Timmons et al. 1996). Acetylcarnitine, the principal product of the CrAT reaction, makes up greater than 80% of the total acylcarnitine metabolite pool in skeletal muscle and plasma (Muio et al. 2012). It has long been known that intense physical activity

dramatically induces skeletal muscle acetylcarnitine accumulation (Sahlin et al. 1990, Hiatt et al. 1989, Putman et al. 1993). However, the physiological relevance of CrAT activity and acetylcarnitine efflux during exercise remains unknown. Data herein suggest that CrAT-mediated skeletal muscle uptake and/or production of acetylcarnitine might be an important component in exercise. We demonstrate that CrAT functions to offset the acetyl-CoA deficit during high intensity contraction. Indeed, CrAT deficiency resulted in a sharp decline in acetyl-CoA levels after exercise while acetylcarnitine followed the opposite pattern (Figure 17A-B). Intriguing is why CrAT is so important for maintaining acetyl-CoA balance during exercise.

One possibility is that CrAT-mediated uptake of acetylcarnitine might provide an acetyl group donor for the TCA cycle. In support of this hypothesis, we demonstrated an accumulation in acetylcarnitine after exercise in CrAT deficient mice (Figure 17B). Further, we observed a rise in CrAT-dependent uptake and oxidation of acetylcarnitine during exercise (Figure 18D). These data suggest that skeletal muscle clears acetylcarnitine during work. CrAT-mediated mitochondrial acetylcarnitine consumption might also provide a kinetic advantage over alternate fuels. Thus acetylcarnitine might bypass the well-controlled multi-step glycolysis and β -oxidative pathways during high energy demand, providing a rapid one-step substrate for the TCA cycle. In support of this hypothesis, exogenous acetylcarnitine enhanced force generation, delayed fatigue and offset

energy stress in CrAT^{fl/fl} but not CrAT^{SM-/-} muscles despite the presence of 2 mM glucose (Figure 18E-J). Furthermore, maximal palmitoylcarnitine-supported respiration in permeabilized muscle fibers could be further increased with the addition of 5 mM acetylcarnitine (Figure 18C). Conversely, acetylcarnitine did not have the same impact on maximal pyruvate-supported respiration (data not shown). However, factors limiting pyruvate production and/or diverting pyruvate into an anaplerotic substrate could dramatically reduce the pyruvate contribution to intramitochondrial acetyl-CoA during high intensity exercise. Despite evidence supporting this hypothesis, circulating acetylcarnitine, present in micromolar concentrations, accounts for less than 1% of the ATP generating potential of circulating glucose, which is present in millimolar concentrations. Thus, it is surprising that circulating acetylcarnitine could be a factor in skeletal muscle ATP generation.

Therefore, we considered the possibility that intramuscular acetylcarnitine production might contribute a local acetyl group pool for ATP generation. We hypothesize that acetylcarnitine production in oxidative fibers might expand the carnitine pool to benefit glycolytic fibers within the same muscle tissue. During intense exercise, oxidative fibers reach maximal ATP generation well before glycolytic fibers and might begin to efflux a local pool of acetylcarnitine for glycolytic fiber consumption. CrAT protein is most abundant in oxidative skeletal muscle (Figure 15A) but mitochondrial CrAT tracked closely with type IIa,

intermediate fibers (Figure 15B). Therefore, inner-fiber cross talk within type IIa fibers might add to the acetyl group pool, though more work is needed to fully elucidate this mechanism.

However, recent studies using magnetic resonance spectroscopy in working heart suggest a more sophisticated mechanism of action. Schroeder and colleagues observed that 50% of pyruvate-derived citrate cycled through acetylcarnitine before entry into the TCA cycle (Schroeder et al. 2012). Therefore, we considered the possibility that flux through the CrAT reaction might be thermodynamically advantageous during high intensity exercise. Thus cycling through CrAT could be critical for acetyl-CoA trafficking from PDH production into the TCA cycle. Because acetyl-CoA is a potent inhibitor of PDH activity (Sugden et al. 2003), CrAT might function to keep the concentration gradient in favor of acetyl-CoA production during exercise (Figure 20). This theory is reminiscent of the creatine kinase and adenylate kinase phosphotransfer reactions. The adenylate kinase reaction combines ADP to produce ATP and AMP. AMP is then degraded to IMP in order to keep the adenylate kinase reaction favoring the production of ATP. Likewise, the creatine kinase reaction is critical for the rapid and efficient trafficking of ATP from its production site in the mitochondria to the myofibrils during work. In this way, creatine kinase prevents sluggish ATP diffusion to contractile machinery and avoids feed-back inhibition of ATP production. In aggregate, exercise stimulation requires these coupled reactions

for the rapid and efficient transfer of energy. Likewise, we suggest the requirement of CrAT for acetyl group trafficking within the mitochondria.

Carnitine supplementation in CrAT^{fl/fl} but not CrAT^{SM-/-} mice improved exercise performance (Figure 19), suggesting that expansion of the skeletal muscle carnitine pool during contraction might stimulate CrAT cycling. However, though circulating carnitine and acetylcarnitine levels were elevated with exogenous carnitine provision, skeletal muscle levels remained unchanged (Figure 19E-F). We hypothesize that elevated carnitine uptake during contraction accounts for the stimulation in CrAT activity while exercise cessation results in metabolite efflux. In support of this hypothesis, data herein demonstrated a stimulation-induced rise in skeletal muscle acetylcarnitine influx and oxidation (Figure 18D). Furthermore, studies in skeletal muscle have shown a contraction-mediated boost in carnitine uptake (Furuichi et al. 2012). Therefore, expansion of the muscle carnitine pool during exercise might stimulate CrAT activity, providing thermodynamically favorable acetyl-CoA trafficking.

We propose the importance of CrAT-mediated acetyl-CoA buffering in disorders wherein exercise intolerance and fatigue lead to inactivity, such as in individuals with peripheral vascular disease (PVD), aging, ischemia and inborn errors of mitochondrial metabolism. Because exercise promotes metabolic health and improves a multitude of maladies, understanding and improving exercise tolerance and fatigue is critical for these patients. For example, PVD skeletal

muscle is associated with decreased work efficiency marked by excessive PCr use and blunted exercise capacity (Greiner et al. 2006). Similarly, aging skeletal muscle is associated with lowered PCr hydrolysis and diminished peak contractile force and ATP production (Tevald et al. 2009, Lanza et al. 2007, Campbell et al. 1991). This phenotype results in compromised exercise tolerance, leading to inactivity and accelerated cardiometabolic decay. Therefore, improved energy efficiency through stimulation of the CrAT reaction might be key for the treatment and prevention of exercise intolerance. In support of this hypothesis, provision of both carnitine and propionylcarnitine improved walking distance in PVD patients (Brevetti et al. 1992, Brevetti et al. 1988). These data suggest that strategies aimed at stimulating flux through the CrAT reaction could prove beneficial for promoting energy metabolism and delaying fatigue.

5 Conclusions and Future Directions:

Data herein focused on the CrAT enzyme and its role in regulation of the intramitochondrial acetyl-CoA pool. We presented compelling evidence suggesting that maintenance of the acetyl-CoA pool is imperative for mitochondrial homeostasis. Chapter 3 summarized the negative consequences of acetyl-CoA overproduction and proposed the important role of CrAT in preventing accumulation of this metabolite. These data suggest that lipid induced inhibition of CrAT activity, resulting in a decrease in the skeletal muscle acetylcarnitine:acetyl-CoA ratio, contributes to insulin resistance. Alternatively, Chapter 4 suggested the deleterious effects of an acetyl-CoA deficit during exercise. These studies demonstrate a role for CrAT in acetyl-CoA buffering and provide insight into exercise physiology, potentially providing important implications for patients with muscle weakness and fatigue. In aggregate, these data suggest a critical role for CrAT as a regulator of mitochondrial acetyl-CoA. Because CrAT is highly involved in fine-tuning both acetyl-CoA accumulation and deficiency, a model we are just beginning to understand, it is likely that we have not yet fully elucidated its role in mitochondrial energetics. Moreover, it seems feasible that remaining questions about the regulation of CrAT activity will provide important insights into the pathogenesis of multiple disease states. Though progress has been made to understand the role of CrAT in metabolism,

many questions remain. The following sections contain an overview of several unanswered questions pertaining to the involvement of CrAT in mitochondrial homeostasis.

5.1 Reactive Oxygen Species:

Multiple studies have demonstrated an antagonistic role of fat load on insulin signaling and glucose uptake (Hirosumi et al. 2002 and Shulman et al. 2000). These data suggest that impaired insulin action occurs by diverting fatty acids away from mitochondrial oxidation, leading to the buildup of lipid species capable of inhibiting insulin signaling. However, recent studies observed lipid-induced derangements in substrate switching within isolated mitochondria (Noland et al. 2009, Koves et al. 2008), suggestive of a connection between mitochondrial bioenergetics and insulin action. Genetic and nutritional models of obesity and diabetes led to excessive β -oxidation and metabolic inflexibility, while genetic inhibition of fat oxidation preserved glucose tolerance (Noland et al. 2009, Koves et al. 2008, Koves et al. 2005). These studies suggest that derangements in mitochondrial metabolism might compromise insulin action, though the link between mitochondrial impairment and insulin signaling is poorly understood. Recent evidence suggests that insulin signaling may be negatively affected by mitochondrial-derived reactive oxygen species (ROS) formation (Bloch-Damti and Bashan 2005, Evans et al. 2005). ROS production has gained momentum as a factor in the development of insulin resistance (Anderson et al.

2007, St-Pierre et al. 2002). ROS have been shown to activate multiple serine kinases and transcription factors implicated in diminished insulin signaling (Bloch-Damti and Bashan 2005, Chakraborti and Chakraborti 1998). As previously described, ROS production is high when ATP demand is exceeded by electron flux into the ETC. This causes heightened backpressure on complex I, resulting in increased electron leak and ROS production (reviewed in Fisher-Wellman and Neuffer 2012, Muoio and Neuffer 2012). ROS can then interact with glutathione, the primary redox buffer in the cell, shifting the ratio of reduced glutathione (GSH) to its oxidized counterpart (GSSH). Just four hours after a single meal high in fat, ROS emission more than doubled while a 50% reduction in the GSH/GSSG ratio was observed in rat skeletal muscle fiber bundles, owing to the sensitivity of complex I electron leak to the redox state. Moreover, 5 days of high fat diet resulted in a persistent oxidative environment due to sustained elevation in ROS. This shift in redox environment toward the oxidative state could provide a potential link between insulin resistance and mitochondrial ROS (Anderson et al. 2009). In support of this hypothesis, interventions that lowered mitochondrial ROS production were shown to preserve insulin action (Lee et al. 2010, Hoehn et al. 2009, Anderson et al. 2009). Therefore, understanding the development and maintenance of ROS could provide critical information regarding the pathogenesis of insulin resistance.

Preliminary data suggests that CrAT might play a role in mitigating the production of ROS. We used confocal microscopy to establish that carnitine treatment reduced the production of ROS by 60% in human skeletal muscle cells (figure 21).

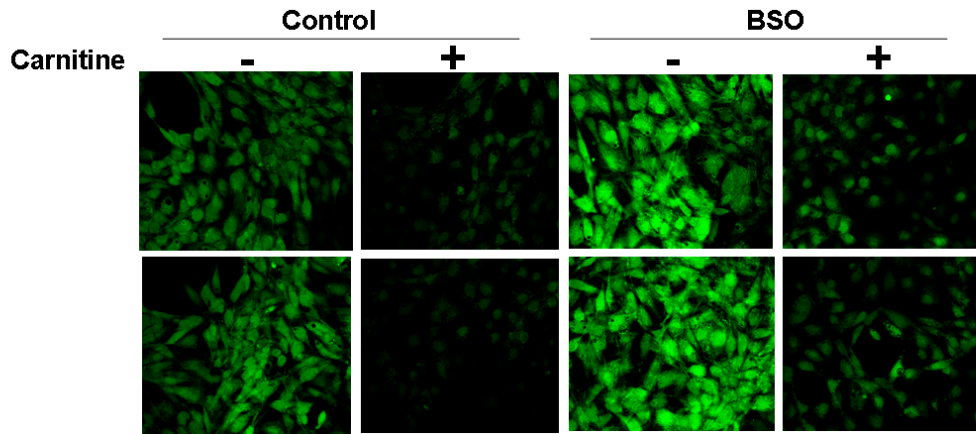


Figure 21: Carnitine Mitigates Production of Reactive Oxygen Species in Cultured Myocytes

Dichlorofluorescein diacetate (DCFDA) treated L6 cells were given 100uM carnitine during differentiation and 100µM buthionine sulfoximine (BSO) for 24 hours where indicated and imaged on a Leica SP5 confocal microscope. BSO selectively inhibits the synthesis of glutathione, which results in increased production of reactive oxygen species.

These data suggest a possible link between heightened CrAT activity and reduced ROS production. Through its unique ability to efflux excess carbons, CrAT may alleviate mitochondrial stress in situations of high fuel load and low energy demand, such as during nutrient overload or immediately upon the cessation of exercise. Therefore, manipulations designed to stimulate CrAT

activity, such as carnitine supplementation, might reduce mitochondrial stress by re-routing short chain acyl-CoA surplus into carnitine esters.

Strong evidence shows that mitochondrial ROS generation is increased immediately following contractile activity. Because ROS production is elevated when ATP demand is low (physical inactivity) but nutrient delivery is high (overfeeding or immediately post-exercise), we hypothesize a potential importance of the CrAT enzyme in relieving mitochondrial stress after contraction. Succinyl-CoA, which is uniquely positioned as the only acyl-CoA intermediate of the TCA cycle, also donates reducing equivalents into the ETC via succinate. We propose that cessation of exercise results in an abrupt drop in ATP production and electron transport while flux through the TCA cycle remains high. Therefore, increased succinate dehydrogenase-derived reducing equivalents have the potential to increase ROS production through backpressure on complex I. We have shown that CrAT acts preferentially on short chain acyl-CoAs (Chapter 3). Because succinyl-CoA is a four carbon short chain acyl-CoA, we believe it to be a substrate for the CrAT reaction. In support of this hypothesis, recombinant adenovirus technology overexpressing CrAT in primary human skeletal muscle myocytes resulted in a 12 fold elevation in skeletal muscle succinylcarnitine (Figure 22A). Additionally, skeletal muscle specific CrAT deficiency resulted in a 50% reduction in succinylcarnitine metabolite levels at

rest, 10, and 60 minutes after a short term high intensity exercise bout (Figure 22B).

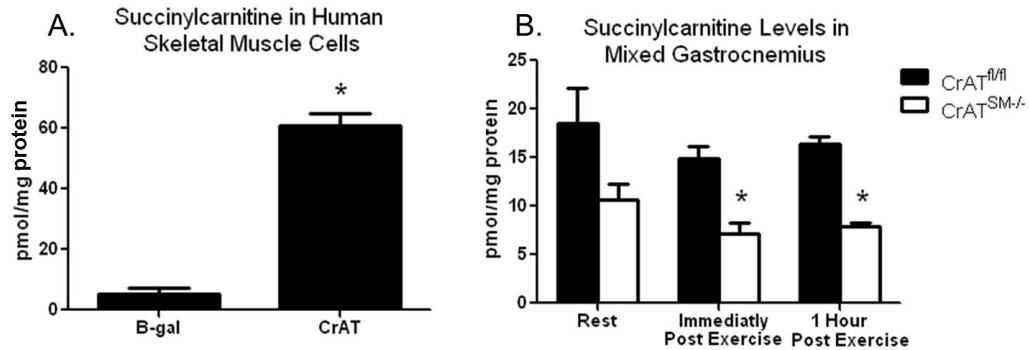


Figure 22: Manipulations in CrAT Alter Skeletal Muscle Succinylcarnitine Content

Acylcarnitine levels were measured in primary human skeletal muscle cell lysate after treatment with B-gal control or rat CrAT overexpression adenovirus (A). CrAT skeletal muscle specific knockout mice (CrAT^{SM-/-}) were subjected to an 11 minute high intensity exercise bout. Data was collected at rest, immediately following exercise and 1 hour post exercise in CrAT^{SM-/-} compared to control mice (CrAT^{fl/fl}) (B).

We hypothesize that CrAT plays a role in relieving the exercise-mediated build-up of succinyl-CoA by forming succinylcarnitine, thus reducing the production of ROS following an exercise bout. In support of this theory, we used metabolomic profiling to demonstrate that plasma levels of succinylcarnitine increased after a short term, high intensity exercise bout in wild type mice but not in mice with a skeletal muscle specific deletion of CrAT (figure 23).

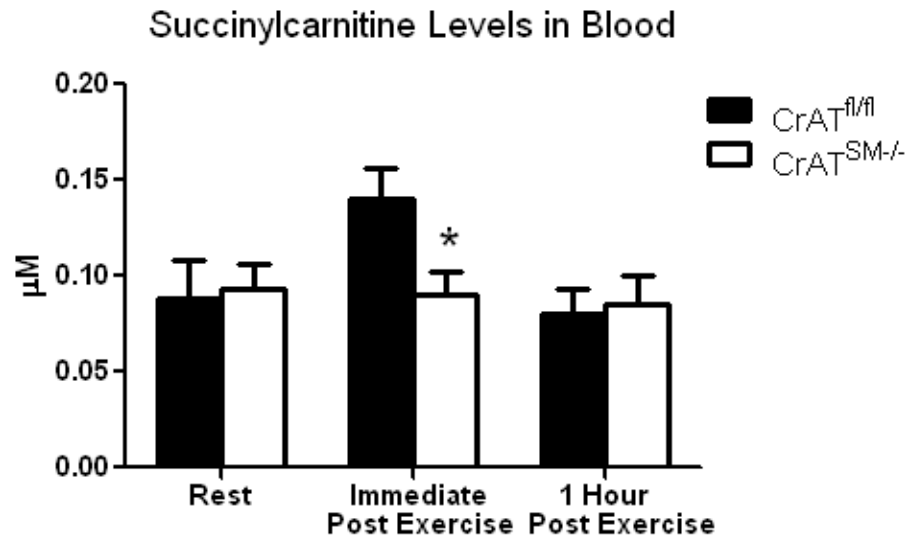


Figure 23: CrAT Deficiency Alters Exercise-induced Changes in Circulating Levels of Succinylcarnitine

Acylcarnitine levels were measured in CrAT^{fl/fl} and CrAT^{SM-/-} mice subjected to an 11 minute high intensity exercise bout. Blood was sampled at rest, immediately following exercise and 1 hour post-exercise.

To definitively test the importance of CrAT in preventing ROS production, ROS indicators such as skeletal muscle glutathione levels, lipid peroxides, and protein carbonyls must be analyzed in CrAT skeletal muscle knock out and wild type littermates both before and after an exercise challenge. Additionally, ROS signaling pathways should be assessed via western blot analysis. Lastly, amplex red can be used to measure ROS generation in isolated skeletal muscle mitochondria from wild type and CrAT deficient mice. These studies will provide

novel insights into the role of CrAT in regulating the mitochondrial redox environment.

5.2 Lysine Acetylation:

Lysine acetylation, a reversible post-translational modification, has become increasingly recognized as a relevant link between fuel metabolism and mitochondrial stress. Sirtuins, a family of nicotinamide adenine dinucleotide (NAD⁺)-dependent protein deacetylases have been shown to regulate the acetylation state of several key metabolic enzymes. Seven sirtuins have been identified with varied compartmentalization. SIRT1, SIRT6 and SIRT7 are found in the nucleus, SIRT1 and SIRT2 in the cytoplasm and SIRT3-5 in the mitochondria (reviewed in Denu 2005). Within the mitochondria, SIRT3 appears to be the primary deacetylase, with no observable deacetylase activity of SIRT4 or SIRT5. Robust mitochondrial hyperacetylation was observed in SIRT3 knockout mice, while no change was obvious in knockouts of either SIRT4 or SIRT5 (Lombard et al. 2007). Interestingly, SIRT5 has been shown to drive mitochondrial desuccinylase and demalonylase activity (Du et al. 2011), while the primary role of SIRT4 is still under investigation.

The NAD⁺ dependent activation of sirtuins suggests a link between deacetylase activity and the energy status of the cell. Supporting this hypothesis, an estimated 35% of mitochondrial proteins have shown to be acetylated, the majority of which are involved in energy metabolism (Anderson and Hirschey

2012). Moreover, greater than 50% of proteins involved in glucose, fatty acid, and amino acid oxidation as well as the TCA cycle and oxidative phosphorylation were found to be acetylated (Anderson and Hirschey 2012), highlighting the potential importance of mitochondrial acetylation state on fuel metabolism. Found to be highly expressed in metabolically active tissues such as liver, heart and oxidative skeletal muscle, SIRT3 activity is induced during fasting, caloric restriction, and exercise training (Palacios et al. 2009, reviewed in Nogueiras et al. 2012). These data imply a critical role for SIRT3 in stimulating mitochondrial fuel metabolism during low energy stress. On the other hand, SIRT3 knockout mice exhibited accelerated glucose intolerance with age and high fat diet (HFD), suggesting a link between aberrant SIRT3 activity and the metabolic syndrome (Hirschey et al. 2011). Interestingly, though an acute high fat diet induced SIRT3 expression (Bao et al. 2010), chronic nutrient overload led to decreased SIRT3 protein and mRNA in both liver (Hirschey et al. 2011, Bao et al. 2010) and skeletal muscle (Jing et al. 2011), resulting in mitochondrial hyperacetylation. The loss of SIRT3 seen in HFD-fed mice is thought to be driven by decreased activity of peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α), a transcriptional coactivator involved in energy metabolism which is suggested to target SIRT3 (Kong et al 2010, Hirschey et al 2011). In support of this hypothesis, acute HFD stimulated while chronic HFD compromised PGC-1 α mRNA (Koves et al. 2005), similar to the observed nutritional regulation on

SIRT3. Furthermore, PGC-1 α was shown to interact with the SIRT3 promoter (Kong et al. 2010), and exogenous PGC-1 α rescued the HFD-induced loss in SIRT3 (Hirschey et al. 2011).

While extensive research has gone into deacetylase activity through sirtuin action, little is known about mitochondrial acetylase activity. Abundance of acetyl-CoA, the common metabolic intermediate of glucose, fatty acid and amino acid oxidation, has been suggested to positively correlate with hyperacetylation (Hirschey et al. 2011, Schwer et al. 2009). In support of this hypothesis, we have shown a significant increase in skeletal muscle acetyl-CoA as a result of a 20 week high fat diet (Chapter 3), which is consistent with decreased SIRT3 abundance and elevated protein acetylation (Jing et al. 2011). However, the mechanism driving acetylation is not fully elucidated. Though mitochondrial acetyltransferases have not been identified, their activity could prove to be important in the regulation of mitochondrial acetylation state. Non-enzymatic acetylation has also been shown to occur *in vitro* on histones (Paik et al. 1970), suggesting the possibility that acetyl-CoA levels could drive either enzymatic or non-enzymatic acetylation.

Because acetyl-CoA is the primary substrate for the CrAT reaction, we propose a role for CrAT in regulating mitochondrial acetylation. Recent studies have demonstrated that CrAT deficiency, shown to induce acetyl-CoA accumulation (Chapter 4), resulted in a glucose intolerant phenotype (Muoio et

al. 2012), likely due in part to persistent acetyl-CoA-mediated PDH inactivation. However, we propose CrAT ablation additionally leads to mitochondrial hyperacetylation driven by elevated acetyl-CoA, potentially further dysregulating metabolic flexibility. Moreover, because flux through the CrAT reaction is diminished in high fat diet and diabetic rodents, models with observed hyperacetylation (Chapter 3, Hirschey et al. 2011, Jing et al. 2011), we suggest a critical role for CrAT in governing acetyl-CoA levels when nutrient load exceeds energy demand. Therefore, we propose CrAT may function to promote metabolic flexibility and glucose tolerance through the efflux of carbon fuels, thereby curbing acetyl-CoA-mediated hyperacetylation.

Preliminary data supporting this hypothesis has been generated by two postdoctoral fellows in the Muoio lab. Drs. Michael Davies and Lilja Kjalarsdottir found elevated mitochondrial acetylation in CrAT deficient skeletal muscle in mice fed a high fat diet (unpublished data). Further, proteomics screening resulted in an observed 14 hyperacetylated mitochondrial proteins in CrAT deficient skeletal muscle compared to wild type controls (unpublished data). These data suggest a role for CrAT in mitochondrial acetylation state. However, more work is required to elicit a mechanism. Use of the CrAT skeletal muscle-specific knockout mouse coupled with site directed mutagenesis will potentially uncover interactions between CrAT regulation and enzyme control in the mitochondria. Additionally, these tools could become important in identifying

acetylation-targeted proteins that may be independent of SIRT3 regulation. These studies will improve our understanding of interactions between acetylation state and the metabolic syndrome.

5.3 Alzheimer's Disease and Acetylcarnitine: Potential role for CrAT in Neurodegenerative Disease.

Alzheimer's disease (AD), the most common form of dementia, is projected to affect 66 million people by 2030 and a staggering 115 million by 2050 (Alzheimer's disease international consortium). AD is classified by the presence of neurofibrillary tangles (aggregates of the hyperphosphorylated tau protein), and amyloid β peptide plaques in the brain (Braak and Braak 2001). The need to find underlying mechanisms of disease is high, as the Medicare and Medicaid spending for AD patients was \$130 billion in 2011 and continues to rise. Moreover, healthcare costs associated with AD patients are three and nine times higher than non-AD patients through Medicare and Medicaid, respectively (Bynum 2011).

Two forms of AD exist, early onset (with primarily genetic risk factors), and late onset. Strikingly, late onset AD is prevalent in more than 50% of people over the age of 85. Genetic risk factors identified in early onset AD include mutations in three genes, amyloid- β protein precursor, and both presenilin 1 and 2 (Selkoe et al. 1997). Genetic studies have also identified risk alleles for late onset AD,

including disruptions in the APOE- ϵ 4 allele. Mutations in APOE- ϵ 4 lead to a three times increased risk of developing AD (Farrer et al. 1997). Importantly, the advent of genome wide association studies has identified nine additional risk genes (reviewed in Bettens et al. 2013), with more likely to come as next-generation sequencing broadens the potential to probe for variants. Importantly, alternative lifestyle risk factors have been reported. These include age, lower education (Cummings 2004), and the metabolic syndrome (reviewed by Luchsinger and Gustafson 2009). Despite progressing work to delineate AD risk factors, the underlying mechanisms of disease are still largely unknown.

Though drug treatments exist for cognitive and behavioral AD symptoms, there is no cure for the disease. One such intervention that has gained recent attention is acetylcarnitine supplementation. Acetylcarnitine has been shown to slow the progression of neurodegeneration and improve spatial learning, memory function and other cognitive and behavioral measurements (reviewed in Liu and Ames 2005 and Acetyl-L-carnitine, monograph 2010). Initially, acetylcarnitine was expected to act through choline acetyltransferase to combine acetyl-CoA with choline to produce the neurotransmitter, acetylcholine (Imperato et al. 1991, Dolew and Tucek 1981). However, studies showing the positive effects of acetylcarnitine on energy metabolism suggest a more sophisticated mechanism of action. We hypothesize that acetylcarnitine, the primary substrate of the reverse CrAT reaction, might be acting through neuronal CrAT to buffer

intramitochondrial acetyl-CoA in AD patients, thereby increasing energy metabolism and brain function. In support of this hypothesis, CrAT has been shown to be highly active in the brain (Bailey and Lahiri 2012), with over 90% greater activity compared to choline acetyltransferase (Kalaria et al. 1992). However, neuronal CrAT activity appears to diminish with age (Liu et al. 2002b). Moreover, patients with AD have further decreased CrAT activity in the frontal cortex, temporal cortex, hippocampus and cerebellum compared to age-matched control subjects (Kalaria et al. 1992), suggestive of a role for CrAT in AD.

Though the mechanism underlying acetylcarnitine action is unknown, it has been suggested that one role of acetylcarnitine supplementation is to delay mitochondrial decay due to mitigation of oxidative damage (Butterfield et al. 2002, Butterfield et al. 2002b, Hagen et al. 2002, Liu et al. 2002). Malondialdehyde (MDA), a product of lipid peroxidation, is significantly increased with age (Liu et al. 2002b), and is induced to a greater extent in Alzheimer's disease compared to control subjects (Ozcanakaya and Delibas 2002). Liu et al. (2002b) showed MDA functions to inhibit substrate binding to the CrAT enzyme. Importantly, acetylcarnitine supplementation restored neuronal CrAT activity and substrate binding. These data imply that the positive effects of acetylcarnitine might be due to activation of the CrAT reaction.

Additional studies suggesting the potential use of acetylcarnitine as a neuronal fuel during starvation (Kuratsune et al. 1997, Yamaguti et al. 1996) may

point to another role for CrAT-mediated acetylcarnitine use in AD. Importantly, acetylcarnitine supplementation in aged rats resulted in decreased neuronal lactic acid coupled with increased phosphocreatine and ATP levels (Aurelit et al. 1990), thereby suggesting a similar CrAT-mediated acetyl-CoA buffering mechanism as described in skeletal muscle in Chapter 4. Furthermore, diminished ATP levels in a neuronal AD cell line were rescued with acetylcarnitine supplementation (Dhitavat et al. 2002). We hypothesize that the critical role of acetylcarnitine in buffering acetyl-CoA and ATP is blunted by diminished CrAT activity in the aging brain and is further disrupted in patients with AD, contributing to neuronal derangements associated with disease. Therefore, the study of neuronal CrAT could prove fruitful for the treatment of AD. To definitively test the importance of CrAT in AD, studies using our tamoxifen-inducible total body CrAT knock out mouse model could become useful. These studies in combination with acetylcarnitine supplementation in aging mice might enhance our understanding of acetylcarnitine supplementation and the pathogenesis of AD.

5.4 Summary:

In summary, we proposed the critical role of CrAT in acetyl-CoA regulation. These studies demonstrated that alterations in this metabolite pool are detrimental to mitochondrial homeostasis. Summarized herein, these findings

additionally suggest the potential role of CrAT in mitochondrial stress and neuronal nutrient homeostasis.

Data herein demonstrated an inhibitory role of long chain acyl-CoAs on forward flux through the CrAT reaction. Lipid stress may persistently compromise CrAT activity in obesity and diabetes, leading to a potentially harmful accumulation of acetyl-CoA which contributes to derangements in metabolic flexibility. In support of this hypothesis, muscle specific CrAT deficiency resulted in glucose intolerance and a metabolically inflexible state (Muoio et al. 2012). These data suggest that a small molecule inhibitor of the long chain acyl-CoA binding site on CrAT may be a useful treatment for insulin resistance. This treatment would allow increased flux through the CrAT reaction and normalized glucose homeostasis. Because incidence of diagnosed cases of type II diabetes have nearly doubled in the past decade, (CDC 2010), treatment options that normalize glucose homeostasis before progression to full-blown type II diabetes are critical.

Alternatively, Chapter 4 summarized the role of CrAT in buffering acetyl-CoA during exercise. These studies demonstrated the role of CrAT-mediated acetyl-CoA maintenance in fatigue. We hypothesize that CrAT functions to sustain high rates of mitochondrial ATP production in two ways. First, contraction-induced recycling and/or import of acetylcarnitine could supply a readily available source of acetyl group donors to buffer transient deficits in

rates of glucose, amino acid and fatty acid catabolism. Secondly, CrAT permits rapid and efficient trafficking of acetyl groups from production to the TCA cycle. By residing in close proximity to the various enzymatic sources of acetyl-CoA (e.g. PDH and ketothiolase), CrAT might alleviate kinetic hindrances due to product inhibition. Additionally, similar to creatine kinase, which uses PCr for the trafficking of ATP from its production site to the myofibrils during work, we hypothesize that cycling through the CrAT reaction could be critical to overcome the thermodynamic inefficiencies of diffusional flux. These studies could provide therapeutic insights into patients suffering from muscle weakness and fatigue, commonly seen with inborn errors of mitochondrial metabolism, peripheral vascular disease and cardiometabolic disease.

In aggregate, these data highlight the importance of acetyl-CoA buffering for the maintenance of PDH activity and glucose disposal. We suggest that lipid-induced regulation of the CrAT enzyme promotes the transition from feeding to fasting. However, this mechanism seems counterintuitive during exercise, when long chain acyl-CoA concentration is high (Thyfault et al. 2010) but flux through the CrAT reaction is critical. Similarly, palmitoylation has been shown to inhibit multiple enzymes in glycolysis during the fed to fasted transition, whereas this regulation would become detrimental during exercise (Yang et al. 2005, Jenkins et al. 2011). One possible explanation is that during exercise, enzyme substrates overwhelm the CrAT reaction, overriding any inhibition by long chain acyl-CoA.

This scenario does not seem likely because palmitoyl-CoA acts as a mixed-model inhibitor of CrAT. The term “mixed” refers to the combination of uncompetitive inhibition, where the inhibitor can bind only to the enzyme-substrate complex and competitive inhibition, where the inhibitor can bind to the enzyme only in the absence of substrate. Therefore, palmitoyl-CoA-mediated inhibition affects the CrAT reaction regardless of short chain acyl-CoA abundance. Rather, we suggest the possibility that rapid turnover of the long chain acyl-CoA pool during exercise might prevent CrAT inhibition. Immediate utilization of the long chain acyl-CoA pool reduces the likelihood that excess palmitoyl-CoA might accumulate and inhibit CrAT. Likewise, rapid turnover of long chain acyl-CoAs would prevent accumulation of the palmitoyl-CoA necessary for the palmitoylation and inhibition of enzymes in glycolysis. Therefore, we propose the importance of fatty acid turnover in the regulation of CrAT activity. This hypothesis would suggest a fatty acid-mediated inhibition of CrAT at rest but not during exercise.

However, data herein demonstrate a persistent inhibition of the CrAT enzyme in obese and diabetic rodents. These derangements in CrAT activity could lead to exercise intolerance. Indeed, aging and chronic disease are often accompanied by muscle weakness and fatigue. These patients could be experiencing chronic lipid-induced CrAT insufficiency, contributing to exercise intolerance. Because exercise promotes metabolic health and mitigates a wide

range of medical conditions, stimulation of the CrAT reaction in these patients could delay fatigue and promote exercise capacity. However, more work is needed to fully understand this mechanism.

Though the current document discusses multiple novel aspects of the CrAT enzyme activity, more work is needed to fully elucidate its action. We propose this enzyme could have an important role in mitigating reactive oxygen species production and hyperacetylation. Additionally, we believe CrAT could play a role in degenerative disorders, such as Alzheimer's disease in the brain. Though the answer to these and a number of other intriguing questions await further investigation, data herein provide a better understanding of CrAT action and suggests multiple paths of therapeutic intervention.

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Absence of Malonyl-CoA Decarboxylase (MCD) Impacts Endurance Exercise Capacity and Reprograms Skeletal Muscle Mitochondrial Metabolism. (In preparation).

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Polymorphisms Associated with Mitochondrial DNA of Elite Kenyan Endurance Runners. (In preparation).

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Honors and Awards

Duke University

Keystone Symposium Conference Assistant 2011

DUMC Department of Pharmacology and Cancer Biology Fitzgerald

Outstanding Poster Award 2009

Lehigh University

Lehigh University Merck Chemistry award 2007

National Society of Collegiate Scholars 2003-2007

Academic All-American, NCAA 2003-2007