Abstract

Effect of Acute Exercise or Fasting on Mitochondrial Function and High Fat Diet-Induced Insulin Resistance

by Oh Sung Kwon

August, 2009

Director: P. Darrell Neufer, Ph.D.

DEPARTMENT OF EXERCISE AND SPORT SCIENCE

A high-fat diet leads to an accumulation of lipid in skeletal muscle, and the development of both mitochondrial dysfunction and insulin resistance. Recently, our lab reported that lipid overload leads to elevated H_2O_2 emission from muscle mitochondria, and that mitochondrial-targeted scavenging of H_2O_2 completely prevents the development of high fat diet-induced insulin resistance. These findings raise the possibility that interventions which acutely restore cellular metabolic balance in muscle may also acutely restore insulin sensitivity. We hypothesized that mitochondrial function and insulin sensitivity can be restored in skeletal muscle of high-fat fed rats by creating an acute deficit in metabolic balance via 2 h low-intensity treadmill exercise or 16 h fasting. Male Sprague-Dawley rats (125-150g) were either maintained on a standard high carbohydrate- diet or fed a high-fat (60%) diet for 6 weeks and divided into three groups the day before the study: one group was maintained on the normal high-fat diet, another group was fasted overnight (16 h), and a third group completed a single 2 h bout of low-intensity treadmill exercise (10 m/min) and then were given normal overnight ad libitum access to the high-fat diet. Oral glucose tolerance tests were administrated to assess

insulin action. Red gastrocnemius muscles were harvested and permeabilized fibers prepared for determination of mitochondrial respiratory function and H_2O_2 emission. A single 16 h fast significantly ($P<0.05$) improved insulin sensitivity in rats maintained on a high-fat diet ($P<0.05$). Oxygen consumption rate in permeabilized fibers in response to submaximal and maximal ADP concentration when supported exclusively with complex I substrates were not different among groups. However, when respiration was supported by fatty acids (palmitoylcarnitine plus malate, complex I + II substrates), high-fat diet plus exercise group showed higher ($P<0.05$) rates compared with high-fat diet group. There were no significant differences in H_2O_2 emission among the 4 groups. In conclusion, a single 16 h overnight fast is sufficient to restore insulin sensitivity in high fat diet-induced insulin resistant rats, providing evidence that insulin action in muscle is acutely sensitive to the metabolic state of cells. A single bout of low-intensity treadmill exercise in high-fat fed rats failed to restore insulin action but increased ADP-stimulated respiratory capacity, providing evidence of an as yet unidentified regulatory mechanism of the respiratory system. Somewhat surprisingly however, neither fasting nor exercise altered the H_2O_2 emitting potential in permeabilized fibers, suggesting that further work is required to better understand the factors influencing mitochondrial function and their potential link to insulin sensitivity.

©Copyright 2009
Oh Sung Kwon

Effect of Acute Exercise or Fasting on Mitochondrial Function and High Fat Diet-Induced Insulin Resistance

A Thesis

Presented To

The Faculty of the Department of Exercise and Sport Science

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Oh Sung Kwon

August, 2009

Effect of Acute Exercise or Fasting on Mitochondrial Function and High Fat Diet-Induced Insulin Resistance

by

Oh Sung Kwon

APPROVED BY:

DIRECTOR OF THESIS: ______________________________
P. Darrell Neufer, Ph.D.

COMMITTEE MEMBER: ______________________________
Joseph A. Houmard, Ph.D.

COMMITTEE MEMBER: ______________________________
Peter A. Farrell, Ph.D.

COMMITTEE MEMBER: ______________________________
Christopher Wingard, Ph.D.

CHAIR OF THE DEPARTMENT OF EXERCISE AND SPORT SCIENCE:

____
Stacey Altman, J.D.

DEAN OF THE GRADUATE SCHOOL:

____
Paul J. Gemperline, Ph.D.

TABLE OF CONTENTS

LIST OF FIGURES viii

INTRODUCTION 1

REVIEW OF LITERATURE 4

Lipid Metabolism and Insulin Resistance in Skeletal Muscle 4

Mitochondrial Dysfunction and Insulin Resistance 7

Exercise and Regulation of Insulin Sensitivity 9

Summary 11

Hypothesis 12

MATERIALS AND METHODS 13

Animals, High-Fat Diet and Exercise Administration 13

Oral Glucose Tolerance Tests 14

Saponin-Permeabilized Muscle Fiber Bundles 14

Measuring JH_2O_2 in Permeabilized Myofibers 17

Statistical Analysis 18

RESULTS 20

Glucose, Insulin and Index of Insulin Action 20

O_2 Consumption 22

JH_2O_2 in Permeabilized Myofibers 23

DISCUSSION 30

Glucose, Insulin and Index of Insulin Action 30

O_2 Repiratory Function in Permeabilized Myofibers 33

H_2O_2 Emission in Permeabilized Myofibers 35

Summary 36

REFERENCES 37

APPENDIX: ANIMAL CARE AND USE PROTOCOL 46

LIST OF FIGURES

1. Experimental protocols for each group
2. Plasma glucose and plasma insulin concentration in response to oral glucose challenge
3. AUC (arbitrary units) for glucose and for insulin among groups
4. An index of insulin action among groups
5. O_2 consumption rate determined from an ADP titration experiment and O_2 consumption rate supported malate/glutamate, ADP, oligomycin, and FCCP
6. O_2 consumption from a palmitoylcarnitine plus malate experiment
7. Succinate stimulated H_2O_2 emission and palmitoylcarnitine plus malate stimulated H_2O_2 emission

INTRODUCTION

Obesity is now a worldwide epidemic which is associated with a risk of numerous diseases such as type 2 diabetes, hypertension, and dyslipidemia, all of which are related to insulin resistance (Houmard, 2007 and Houmard, 2008). Skeletal muscle is responsible for glucose disposal in response to insulin (Mogensen et al., 2007). The accumulation of lipid in skeletal muscle has been correlated well with the development of insulin resistance (McGarry, 2002) and the reduction in the sensitivity of skeletal muscle to insulin is characterized by a reduced capacity for fatty acid oxidation as well as a lower overall mitochondrial oxidative capacity (Kirkwood et al., 1991). Skeletal muscle of obese individuals is also characterized by altered mitochondria morphology and function as evidenced by reduced size and density of the mitochondria resulting in lower overall respiratory capacity (Houmard, 2008; Kirkwood et al., 1991; Boushel et al., 2007). These findings have led to the suggestion that functional impairment of mitochondria might contribute to the pathogenesis of insulin resistance in skeletal muscle (Kelley et al., 2002).

Physical activity and weight loss are commonly recommended to improve insulin action within the skeletal muscle (Goodpaster et al., 2003). Endurance exercise increases energy demand in muscle thereby increasing the metabolism of both carbohydrate and lipid in muscle. If physical activity is increased daily, intramuscular lipid content decreases and insulin sensitivity improves in muscle (Walsh et al., 2001). Endurance exercise training also increases mitochondrial size, density, and content in skeletal muscle fibers and enhances maximal rates of mitochondrial ATP production (MAPR) (Bruce et al., 2006). Recently, Frederico et al. reported that a

combined dietary weight loss and physical activity intervention improve skeletal muscle electron transport chain (ETC) activity and insulin sensitivity (Frederico et al., 2006). These findings have led to the hypothesis that physical activity improves insulin sensitivity in skeletal muscle by increasing the capacity for fatty acid oxidation which, in turn, lowers intramuscular fatty acid content.

While numerous studies have documented that endurance exercise increases maximal ADP-stimulated mitochondrial respiration, a number of other studies have shown that obesity and inactivity lead to an apparent reduction in mitochondrial function and content in skeletal muscle (Tonkonogi et al., 2000 and Saks et al., 2000). Elevated reactive oxygen species (ROS) is the primary mechanism related to deterioration of mitochondrial function. Skeletal muscle has a number of potential sites for nitric oxide (NO) and superoxide generation, but it also has a well-developed system to control these ROS and prevent deleterious effects (Jackson, 2000). These protective systems involve superoxide dismutase (MnSOD and CuZnSOD, respectively), catalase and glutathione peroxidase enzymes and a number of direct scavengers of ROS including glutathione (Jackson, 2005).

Mitochondria are important sites for the generation and removal of a number of intracellular signaling effectors such as hydrogen peroxide (H_2O_2), and NO. The H_2O_2 emission from mitochondria reflects the balance between the rate of electron leak/superoxide formation from the respiratory system and scavenging of H_2O_2 in the matrix (Stone and Yang, 2006). Once in the cytosol, H_2O_2 can alter the redox state of the cell by either reacting directly with thiol-residues within redox sensitive proteins or by shifting the reduced to oxidized ratio of glutathione (GSH/GSSG), the main redox buffer of the cell. Thus, the rate at which H_2O_2 is emitted from mitochondria is

considered an important barometer of mitochondrial function and modulator of overall cellular redox environment (Schafer and Buettner, 2001). Our laboratory has recently provided evidence that lipid overload causes elevated mitochondrial H_2O_2 emission leading to a shift in redox environment (GSH/GSSG ratio) and a more oxidized state (Anderson et al., 2009). Preventing the increase in H_2O_2 emission through the use of pharmacological and transgenic strategies completely prevented the development of insulin resistance normally induced by a high-fat diet. These findings raise the possibility that insulin sensitivity in muscle is acutely regulated by the cellular redox environment and that interventions that acutely reestablish the cellular metabolic and redox balance may also acutely restore insulin sensitivity.

REVIEW OF LITERATURE

Lipid Metabolism and Insulin Resistance in Skeletal Muscle

The National Health and Nutrition Examination Survey reports that 31% of children who are 6-19 years old were at risk of becoming overweight and 16% were overweight (Hedley et al., 2004). The prevalence of overweight (16%) was more than three times the expected prevalence based on prior surveys (Hedley et al., 2004). This increase in obesity in children and adolescents is undoubtedly a major contributor to the high rates of impaired glucose tolerance, metabolic syndrome, and type 2 diabetes. These diseases are related to insulin resistance.

Insulin is an anabolic hormone essential for the maintenance of glucose homeostasis, tissue growth, and development. It is well known that insulin is secreted by the pancreatic B cells mainly in response to increased blood levels of glucose and amino acids after meals. In addition, the concentration of insulin in the blood displays regular variations independently from food intake (Bergsten, 2000).

Insulin resistance, defined as an inability of target tissues to increase glucose uptake in response to insulin, characterizes virtually all individuals with type 2 diabetes, most of whom are obese. This is particularly relevant to skeletal muscle, because this tissue is responsible for the majority of whole-body insulin-stimulated glucose disposal. The accumulation of lipid within muscle is an aspect of regional fat distribution, which has recently gained considerable attention because of its association with insulin resistance (Goodpaster and Wolf, 2004).

Denton and Randle first studied the existence of lipid storage in muscles (Denton and Randle, 1967). More than three decades later, the association between insulin resistance and triglyceride content measured in human muscle biopsy samples was initially reported by Pan et al. who determined that muscle triglyceride content among non-diabetic Pima Indian men, an ethnic group with a pronounced disposition to obesity and type 2 diabetes, was related to insulin resistance independent of total adiposity (Pan et al., 1997). Since then the relevance of muscle lipid accumulation to insulin resistance in humans has been demonstrated, and basic studies have indicated plausible mechanisms whereby lipid accumulation could generate insulin resistance (Schrauwen, 2007 and Moro et al., 2008).

An association between intramyocellular triglyceride (IMTG) and insulin resistance is now well established in many animal models (Schrauwen, 2007 and Moro et al., 2008). Even in human studies, improved insulin sensitivity achieved by low-caloric diets in patients with type 2 diabetes was accompanied by a reduction in IMTG (Hegarty et al., 2002). However, other studies report that IMTG accumulation is not the whole story. Some lean study participants have substantial muscle insulin resistance without increased IMTG and some high BMI study participants have less IMTG than might be expected for their degree of insulin resistance. But, IMTG is a useful marker of the level of cytosolic lipid accumulation (Schrauwen, 2007 and Moro et al., 2008).

There may be multiple metabolic causes of increased cytosolic lipid accumulation in muscle in insulin-resistant states, with an obvious question as to how much is due to increased uptake versus reduced utilization of fatty acids. In animal studies of insulin resistance with a high-fat diet, a high muscle uptake of fatty acids is

the most significant factor. Rats fed a high-fat diet increase the clearance of fatty acids into muscle (Hegarty et al., 2002). Hegarty and coworkers concluded that lipids accumulate in muscle because the capacity to oxidize fatty acids is out paced by fatty acid uptake (Hegarty et al., 2002). Some studies support a decreased number of mitochondria in muscle from obese individuals with type 2 diabetes (Ritov et al., 2005) and show decreases in gene expression for a number of mitochondrial electron transport proteins (Patti et al., 2003). Other reports suggested changes in ATP production rates and mitochondrial function after prolonged fat feeding but not at the early time points when muscle insulin resistance is already clearly evident in fat-fed rodents (Sparks et al., 2005). Thus, there is an initial upregulation of both fatty acid clearance into muscle, and a subsequent ability to oxidize the fatty acids once in the muscle cell, although the latter is insufficient to prevent cytosolic lipid accumulation in the presence of a high fatty acid supply to muscle. Also, it is possible that there are functional abnormalities in regulatory mechanisms that control muscle triglyceride turnover leading to or contributing to insulin resistance (Moro et al., 2008).

Lastly, exposure to excess fatty acids results in the accumulation of intramyocellular lipid species such as diacylglycerol (DAG) and ceramides. DAG is thought to activate serine kinases that can serine phosphorylate and reduce the signal transduction capacity of insulin receptor substrate 1 (IRS-1). Ceramides can interfere with insulin signaling at the level of Akt and are produced *de novo* from fatty acids or by release from lipids in response to stress cytokines such as tumor necrosis factor (TNF). TNF and other cytokines associated with fat accumulation can also activate serine kinases directly via inflammatory signaling pathways (e.g., c-jun N-terminal kinase, protein kinase c-θ, mammalian target of rapamycin, etc.). Excess fatty acid

oxidation in the mitochondria, via the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC), can lead to an increase in fatty acid metabolites and ROS which can activate intracellular stress kinases but may possibly have effects on insulin signaling to GLUT4 translocation at unknown points downsteam of Akt (Kraegen and Cooney, 2008).

Mitochondrial Dysfunction and Insulin Resistance

Insulin resistance in skeletal muscle is a characteristic of obesity and type 2 diabetes. There is increasing evidence that muscle mitochondrial dysfunction is present in many insulin resistantant states, including type 2 diabetes and obesity (Kelley et al., 2002). Mitochondrial dysfunction may be central to the pathogenesis and the pathophysiology of type 2 diabetes, as it may contribute to insulin resistance as well as to impaired insulin secretion (Petersen et al., 2003; Petersen and Shulman, 2006; Lamson and Plaza, 2002). It has been suggested that reduced muscle mitochondrial activity results in accumulation of intracellular triglyceride that causes muscle mitochondrial dysfunction. So, insulin sensitivity enhances muscle mitochondrial biogenesis, and the activity of the mitochondrial ETC is reduced in the muscles of patients with type 2 diabetes (Petersen and Shulman, 2006).

Mitochondrial oxidative phosphorylation by the ETC provides energy for adenosine triphosphate (ATP) production. However, this also generates ROS. ROS causes damage to DNA, proteins, and membrane structures. An imbalance between increased ROS and decreased endogenous antioxidants within the mitochondria enhances the damaging effects of ROS. Further, ROS may lead to increased mutations

in mitochondrial DNA (mtDNA), which has a limited repair capacity (Maechler and Wollheim, 2001).

Oxidative stress occurs when there is an imbalance between the production of oxidation products and the ability of antioxidant mechanisms to neutralize these products. The production of ROS increases in patients with diabetes (Bayraktutan, 2002). The possible sources for the overproduction of ROS are widespread and include enzymatic pathways, and mitochondria. In diabetes, the overproduction of superoxide has been attributed to an increase in the activity of several enzymes including nitric oxide synthase and NADH/NAD(P)H oxidase. Activation of NAD(P)H oxidase has been also been linked to the increased production of advanced glycation end-production which causes an increase in intracellular formation of H_2O_2 (Wautier et al., 2001).

Several studies reported that mitochondria are a major source for superoxide production in the vasculature of diabetic rats (Coppey et al., 2003). Using cultured bovine aortic endothelial cells, hyperglycaemia increased superoxide production. Hyperglycaemia, due to an overproduction of electron donors derived from glycolysis and the TCA cycle, has been demonstrated to increase the proton gradient across the mitochondrial inner membrane above a threshold level causing a prolonged period of superoxide generation (Korshunov et al., 1997 and Du et al., 2001). It has been shown that overexpression of Mn-SOD abolishes the signal generated by ROS, and overexpression of uncoupling protein-1 collapses the proton electrochemical gradient, thereby preventing the overproduction of ROS by endothelial cells (Nishikawa et al., 2000).

Skeletal muscle has emerged as a target of acute insulin effects on muscle mitochondria in humans. Hyperinsulinaemia in the high physiological range increased transcript levels of complex I and complex II subunits of the respiratory chain (Huang et al., 1999). Interestingly, increments in mitochondrial transcripts were positively related to those of insulin-mediated glucose disposal, supporting the concept that muscle mitochondria are responsive to insulin action to increase fuel utilization. When insulin was infused into healthy men and women, mitochondrial transcript levels, protein synthesis, respiratory chain enzyme activity and the ATP production rate significantly increased after 7 hours. This study demonstrated that insulin is a stimulant of muscle mitochondrial biogenesis and function *in vivo* in humans (Stump et al., 2003).

Exercise and Regulation of Insulin Sensitivity

The most common interventions for the treatment of obesity are increased physical activity and/or reduced caloric intake. Most research has reported that moderate-intensity exercise combined with moderate weight loss induces increases in oxidative capacity of skeletal muscle in obese people (Menshikova et al., 2007). This increase of oxidative capacity of skeletal muscle is associated with improved insulin sensitivity and an increased capacity for fat oxidation.

At rest, the amount of fatty acids released from adipose tissue typically exceeds the amount oxidized; fatty acid rate of appearance into plasma is approximately twice the rate of fatty acid oxidation (Klein et al., 1989). In the case of exercise, mild- or moderate-intensity exercise [25-65% of maximal oxygen consumption (VO_2max)] is

associated with a 5-10 fold increase in fat oxidation above resting amounts because of increased energy requirements of muscle and enhanced fatty acid availability (Krogh and Lindhard, 1920). Moderate-intensity exercise also doubles adipose tissue blood flow, which increases the removal of fatty acids from adipose tissue (Bulow and Madsen, 1976 and Bulow and Madsen, 1981). However, although endurance exercise is commonly found to increase fatty acid oxidation during exercise, an increase in resting fatty acid oxidation after training is not as robust (Horowitz, 2007). It is important to recognize that having an increased capacity to oxidize fatty acids does not necessarily equate to a meaningful increase in fatty acid oxidation at rest, in part because resting energy expenditure is very low (Ross et al., 2000).

Exercise-induced improvements related to skeletal muscle insulin action include increases in GLUT4 protein and activities of hexokinase and glycogen synthase as well as increases in components of the insulin signal pathway (Goodpaster and Brown, 2005). Skeletal muscle triglycerides provide substrate for oxidative energy metabolism during moderate-intensity physical activity. However, there is a discrepancy regarding the effect of endurance training on intramuscular lipid content. Although increased lipid stores are associated with obesity and insulin resistance, endurance-trained athletes, an insulin sensitive population, also have high quantities of IMTG. This may be explained by an enhanced ability to oxidize intramuscular lipids and the use of IMTG as a fuel source during exercise. Thus, although IMTG content may increase, the accompanying elevation in mitochondrial content/oxidative capacity with exercise training may minimize the accumulation of potentially detrimental lipid metabolites such as diacylglycerol, ceramide, long-chain Acyl-CoA (LCA CoA), etc. (Goodpaster and Brown, 2005).

Morphologically, exercise training induces an increase in the size and number of mitochondria [as determined by mtDNA content], adaptations that are largely responsible for the training-induced increases in muscle oxidative capacity (Toledo et al., 2006). Generally, endurance-trained athletes have a higher mtDNA content in muscle than do sedentary individuals, and mtDNA content is proportional to mitochondrial volume density (Puntschart et al., 1995).

Thus, exercise training also shows several factors contribute to this adaptive response: increased density of the mitochondria in the skeletal muscles, which increases the capacity for fat oxidation; a proliferation of capillaries within skeletal muscle, which enhances fatty acid delivery to muscle; an increase in carnitine transferase, which facilitates fatty acid transport across the mitochondria membrane; and an increase in fatty acid binding proteins, which regulate myocyte fatty acid transport (Turcotte et al., 1991 and Turcotte et al., 1999).

Summary

Summarizing the above comments, a high-fat diet leads to an accumulation of lipid in skeletal muscle, and the development of both mitochondrial dysfunction and insulin resistance. In addition, recent evidence from our laboratory indicates that lipid overload leads to elevated H_2O_2 production and emission from muscle mitochondria, resulting in a shift in redox environment (GSH/GSSG ratio) to a more oxidized state. Importantly, scavenging H_2O_2 emission completely prevents the development of high fat diet-induced insulin resistance. These findings raise the distinct possibility that, in high-fat fed rats, interventions which acutely restore cellular metabolic balance in

muscle (i.e., exercise bout, and acute caloric restriction) may also acutely restore intracellular redox environment and insulin sensitivity.

Hypothesis

The purpose of the present study is to examine the interaction between cellular metabolic balance and insulin sensitivity in the context of mitochondrial bioenergetics. We will test the hypothesis that mitochondrial function and insulin sensitivity can be restored in skeletal muscle of high-fat fed rats by creating an acute deficit in metabolic balance via exercise or caloric restriction.

MATERIALS AND METHODS

Animals, High-Fat Diet and Exercise Administration

All animal studies proposed were approved by the East Carolina University Institutional Animal Care and Use Committee. Forty eight male Sprague-Dawley rats weighing 125-150 g were obtained from Charles River Laboratory (Wilmington, MA) and housed in a temperature (22˚C) and light-controlled room and given free access to standard rodent chow and water for one week.

After initial acclimation, rats were housed two to a cage and fed ad libitum either standard high-carbohydrate rodent chow (N=12) or a high-fat diet (~60% calories from fat, #D12492 Research Diets, Inc; N=36) for six weeks. During the final two weeks, rats received 1 ml of saline by oral gavage every other day to familiarize them with the procedure. At the end of the 6th week, one group (controls) remained on standard chow (Figure 1 A). The chronically high-fat fed rats were randomly assigned to one of three groups: the first group (HFD) remained on the high-fat diet, the second group (EX) was subjected to a single bout of low-intensity treadmill exercise (10 m/min) for 2 h (5:30-7:30 p.m.) and returned to their cage with ad libitum access to the high-fat diet, and the third group was fasted for 16 h (beginning at 7:30 pm) (Figure 1 B, 1 C and 1 D). Six rats from each group completed the prescribed protocol and then the following morning, food was removed (4:00 am) and 4 h later the rats were subjected to a 2 h oral glucose tolerance test (OGTT, described below). Once completed, the rats were returned to their cages and remained on their prescribed diet for an additional 7 days. On the 8th day, the same 6 rats repeated the protocol

described above, with the exception that all rats were sacrificed 1 h after receiving the oral glucose gavage. Red gastrocnemius muscle were harvested and frozen for later analysis of activation of insulin signaling proteins (IRS-1, PI3K, and Akt).

During the 7th week, a second set of 6 rats from each group completed the same protocol as described above (acute exercise or 12 h fast). The following morning, all rats were sacrificed and red portions of the gastrocnemius muscle were harvested and permeabilized fibers prepared for determination of mitochondrial respiratory function and H_2O_2 emission (described below).

Oral Glucose Tolerance Tests

On the day of experiments, food was removed 8 h prior to (12:00 am) administration of a 2 g/kg glucose solution via gavage. Thus, all rats completed the initial 4 h of the dark cycle with access to food (with the exception of the 12 h fasted rats). Glucose levels were determined on whole blood samples (Lifescan, Milpitas, CA). Serum insulin levels were determined via a rat/mouse ELISA kit (Linco Research, St. Charles, MO).

Saponin-Permeabilized Muscle Fiber Bundles

Small portions (~10 mg) of right gastrocnemius muscle were separated and placed in ice-cold Buffer X, containing (in mM) 60 K-MES, 35 KCl, 7.23 K_2EGTA, 2.77 CaK_2EGTA, 20 Imidazole, 0.5 DTT, 20 Taurine, 5.7 ATP, 15 PCr, 6.56 $MgCl_2$-$6H_2O$ (pH 7.1, 295 mOsm). The muscle was trimmed of connective tissue and cut down to fiber bundles (~2 x 7 mm, 2-3 mg wet weight). Using a pair of forceps under

a dissecting microscope, fibers were gently separated from one another in order to maximize surface area of the fibers in the bundle (i.e., sarcolemmal surface area is maximized). To permeabilize the myofibers, each fiber bundle was placed in ice-cold Buffer X containing 30 µg/mL saponin and incubated on a rotator for 25-30 minutes at 4°C. The permeabilized fiber bundles (PmFBs) were then washed in ice-cold Buffer Z to remove the extramitochondrial components containing (in mM) 110 K-MES, 35 KCl, 1 EGTA, 5 K_2HPO_4, 3 $MgCl_2$-6H_2O, 0.5 mg/ml BSA, 0.005 glutamate and 0.002 malate (pH 7.4, 295 mOsm) and remained in Buffer Z on a rotator at 4°C until analysis (<45 minutes) without any deterioration in mitochondrial function (i.e., no change in respiratory control ratios).

After washing, one of the fibers was treated with 10 mM sodium pyrophosphate in ice-cold Buffer Z to deplete the fibers of endogenous adenine nucleotides and to prevent Ca^{2+}-independent contraction of the fibers during the assay in parallel with the fluorometric measurements. Both fibers were placed into a separate chamber of the Oroboros O2K oxygraph (Innsbruck, Austria) for high-resolution respirometric measurements. Each chamber had 2 mL of Buffer Z containing 20 mM creatine monohydrate (to saturate endogenous creatine kinase) and 5 mg/mL BSA. Additionally, chamber 2 contained 50 µM *N*-benzyl-*p*-toluene sulphonamide (BTS, an inhibitor of contraction).

Protocol 1: *Oligomycin-induced respiration upon ADP titration in the presence of minimal complex I substrate (glutamate + malate).* This protocol mirrors the succinate titration performed in the fluorometric assay of H_2O_2 production. The minimal complex I substrate (i.e., 5 µM glutamate + 2 µM malate) followed by Oligomycin (inhibits ATP synthase) and carbonylcyanide p-

trifluoromethoxyphenylhydrazone (FCCP, to completely uncouple respiration) to maximally challenge the system.

Protocol 2: *Multiple substrate protocol featuring Palmitoylcarnitine plus malate (PCM), ADP, Glutamate, Succinate, Oligomycin and FCCP*. The first addition (PCM) initiated a state 4 basal, non-phosphorylating respiration (PCM_4). Reducing equivalents are derived only from β-oxidation and the TCA cycle. Maximal ADP is then added to initiate state 3, phosphorylating respiration (PCM_3). Under these conditions, the available substrate concentration is the only limiting factor of respiration. The ratio of state 3 to state 4 respiration (i.e., PCM_3/PCM_4) will give the coupling ratio for PCM. This provides a useful index of 1) the structural integrity of the mitochondrial preparation, and 2) the degree to which respiration is coupled to ATP synthesis. Next, cytochrome *c* is added to indicate an intact outer mitochondrial membrane. The absence of an increase in respiration after addition of cytochrome *c* is enough to conclude that the outer mitochondrial membrane is intact. Addition of 2 mM glutamate will provide a high rate of NADH (complex I substrate) production, and is thus used to test the activity of complex I. The step-increase in oxygen flux upon addition of glutamate is expected to produce the highest rate of O_2 respiration of any substrate in the protocol. Malate in high quantities will equilibrate with fumarate, resulting in inhibition of complex II (succinate dehydrogenase). Therefore, succinate is needed to fully activate Krebs cycle. O_2 consumption is expressed as $pmol \cdot sec^{-1} \cdot mg$ dry $weight^{-1}$.

Measuring JH_2O_2 in Permeabilized Myofibers

We tested the mitochondrial oxidant emitting potential of saponin-permeabilized muscle fibers (~2 mg wet weight each). Fluorescence changes due to Amplex Red oxidation (an indicator of H_2O_2 production) were measured continuously (ΔF/min) using a Spex Fluoromax 3 (Jobin Yvon, Ltd.) spectrofluorometer with temperature control and magnetic stirring at 30°C (Anderson et al., 2009). After establishing background ΔF (de-energized PmFBs in presence of 10 µM Amplex Red, 1U/ml HRP, 10 µg/ml oligomycin, 25 U/ml superoxide dismutase), the reaction was initiated by addition of substrate. H_2O_2 production rate was calculated from the slope of ΔF/min, after subtracting background, from a standard curve established for each reaction condition. The H_2O_2 emission potential of each PmFB was determined fluorimetrically, under state 4 conditions (10 µg/mL oligomycin), by a) titrating the complex II substrate succinate (100 – 3000 µM) in the presence of minimal complex I substrate (glutamate/malate, 5/2 µM); and b) progressively adding saturating concentrations of the following: 25 µM palmitoyl-carnitine + 1 mM malate, 2 mM glutamate, and 3 mM succinate. Addition of 10 µM rotenone was performed at the end of the experiment to show that reverse electron flow at complex I is the source of H_2O_2 emission. The expectation is that the rate of H_2O_2 emission was extinguished upon addition of rotenone.

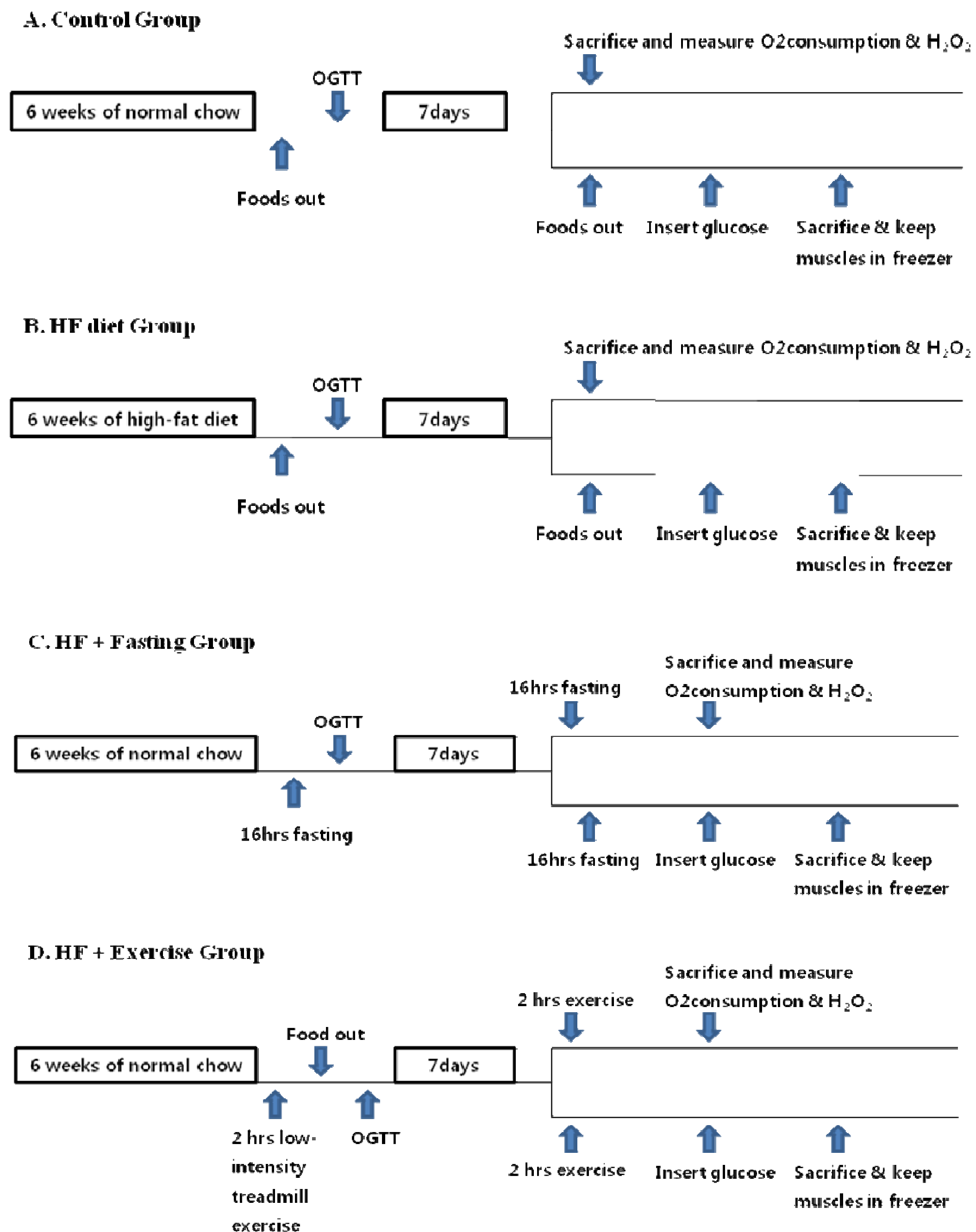
At the conclusion of each experiment, PmFBs were freeze-dried in a lyophilizer (Labconco) overnight for subsequent determination of dry weights of the PmFBs. H_2O_2 production was expressed as $pmol \cdot min^{-1} \cdot mg\ dry\ weight^{-1}$. Both protocols were conducted separately on individual fiber bundles.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analyses were performed using One-way ANOVA with Student-Newman-Keuls method for analysis of significance among groups. The level of significance was set at $P<0.05$.

Figure 1

Experimental protocols for each group; A. Control group, B. High-fat diet group, C. High-fat diet plus overnight fasting group, and D. High-fat diet plus 2 h low-intensity treadmill exercise group.



RESULTS

Glucose, Insulin and Index of Insulin Action

A mismatch between energy supply and energy demand resulting in elevated mitochondrial H_2O_2 emission has recently been implicated in the etiology of insulin resistance induced by a high-fat diet (Anderson et al., 2009). These findings imply that acute interventions, such as overnight fasting or a single exercise bout, may reestablish the balance between intracellular energy supply and energy demand and normalize mitochondrial H_2O_2 emission and insulin sensitivity in skeletal muscle.

To determine if an acute deficit in energy balance can restore insulin sensitivity, rats were placed on a high-fat diet for 6 weeks and then oral glucose tolerance tests were performed under one of three conditions; high-fat diet post-prandial (i.e., food removed 4 h prior to testing), high-fat diet plus overnight fasted (16 h), or high-fat diet plus 2 h low-intensity treadmill exercise bout performed 16 h prior to testing (also 4 h post-prandial). A control group (4 h post-prandial) maintained on a normal high-carbohydrate diet was included for comparison. Blood samples (tail vein) were obtained prior to administration of a 2 g/kg glucose solution via gavage, and at 30 minutes, 60 minutes, and 120 minutes after gavage. Prior to the gavage, glucose level in fasting group (96.58 ± 4.9 mg/dl) was significant lower than the high-fat diet group (114.4 ± 3.2 mg/dl, Figure 2 A). No other differences were noted prior to gavage. As expected, plasma glucose was significantly greater in the high-fat diet group (181.2 ± 10.6 mg/dl) compared with controls (151.5 ± 4.2 mg/dl) 30 min after glucose gavage and remained significantly greater than controls over the

remaining 90 min (Figure 2 A). In the high-fat diet plus fasting group, the plasma glucose response was significantly lower than the high-fat diet group at 30 min (153.3 ± 8.3 mg/dl) and 120 min (159.5 ± 7.9 vs. 196.5 ± 9.9 mg/dl, respectively) after gavage and was not different from control at any time point. In the high-fat diet plus exercise group, the plasma glucose response was significantly lower than the high-fat diet group only at the 120 min time point (150.1 ± 7.9 mg/dl) (Figure 2 A).

Initial serum insulin levels in control (0.2468 ± 0.03 ng/ml), high-fat diet plus fasting (0.1025 ± 0.01 ng/ml), and high-fat diet plus exercise (0.2986 ± 0.03 ng/ml) groups were significant lower than in the high-fat diet group (0.4166 ± 0.05 ng/ml) (Figure 3 B). The increases in serum insulin concentrations at 30 min, 60 min, and 90 min after glucose gavage were significantly lower in the high-fat plus fasting (0.330 ± 0.056 ng/ml, 0.244 ± 0.032 ng/ml, and 0.170 ± 0.015 ng/ml, respectively) than the high-fat diet group (0.7575 ± 0.12 ng/ml, 0.5396 ± 0.07356 ng/ml, and 0.3607 ± 0.044 ng/ml, respectively; $P<0.05$). No other significant differences in insulin responses were noted among the groups.

Area under the curve (AUC, arbitrary units) for glucose and insulin are presented in Figure 3 A and 3 B. AUC for glucose in control (433.5 ± 10.01), high-fat diet plus fasting (465.0 ± 21.79), and high-fat diet plus exercise (473.0 ± 12.55) groups were significant lower than high-fat diet group (545.9 ± 25.72; $P<0.05$). AUC for insulin in high-fat diet plus fasting (0.7105 ± 0.084) was significantly lower than high-fat diet group (1.686 ± 0.22).

To provide an additional indication of insulin sensitivity, each serum glucose and insulin concentration were multiplied and the inverse value was plotted for each time point. AUC for these plots was then taken as an index of insulin action and is

presented in Figure 4. Insulin action (arbitrary units) in control (0.079 ± 0.009) and high-fat plus fasting (0.139 ± 0.015) group was significant higher than high-fat diet group (0.044 ± 0.007; $P<0.05$).

O_2 Consumption

To examine O_2 consumption among groups, data were analyzed by using Oroboros O2K oxygraph (Innsbruck, Austria). Two different protocols were employed. *Protocol 1*: ADP titration in the presence of minimal complex I substrate (glutamate + malate) followed by Oligomycin (inhibits ATP Synthase) and FCCP (completely uncouples respiration), *Protocol 2*: Multiple substrate protocol featuring palmitoylcarnitine plus malate (PCM), ADP, glutamate, succinate, oligomycin & FCCP.

Oxygen consumption rates in response to submaximal and maximal ADP concentrations when supported exclusively with complex I substrates were not different among the 4 groups (Figure 5 A and Figure 5 B).

When respiration was supported by fatty acids (palmitoylcarnitine plus malate, complex I + II substrates), significant differences in respiration capacity were evident between the high-fat diet plus exercise versus the high-fat diet groups (Figure 6). State 3 respiration supported by ADP (217.2 ± 34.93 pmol/s/mg dry wt), glutamate (290.4 ± 46.77 pmol/s/mg dry wt), succinate (452.3 ± 67.52 pmol/s/mg dry wt) and FCCP (585.8 ± 46.17 pmol/s/mg dry wt) in high-fat diet plus exercise group showed higher rates compared with high-fat diet group (ADP: 66.87 ± 21.74, glutamate: 104.5

± 28.11, succinate: 188.1 ± 41.25, and FCCP: 368.3 ± 83.76 pmol/s/mg dry wt; $P<0.05$).

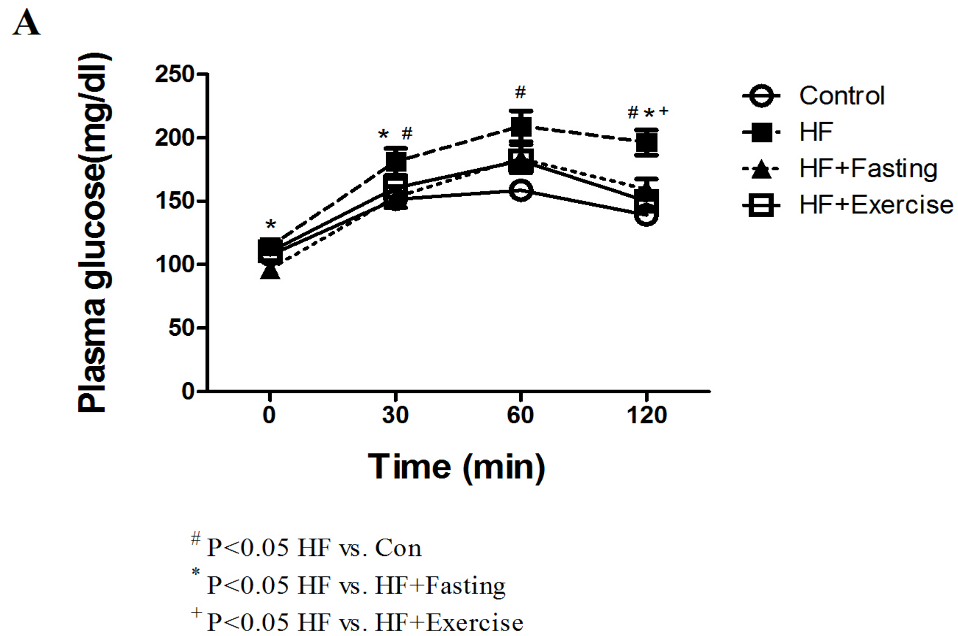
JH_2O_2 in Permeabilized Myofibers

We have previously found that the mitochondrial oxidant emitting potential is increased in permeabilized muscle fibers of rats fed a high-fat diet (Anderson et al., 2009). To determine whether this apparent change in the governance of mitochondrial H_2O_2 emission can be acutely restored by reestablishing metabolic balance, we measured the mitochondrial oxidant emitting potential of saponin-permeabilized red gastrocnemius muscle fibers (~2 mg wet weight each) from control, high-fat diet, high-fat plus fasting, and high-fat plus exercise groups. We used two different protocols: *Protocol 1*; Titrating the complex II substrate succinate (100 – 3000 µM) in the presence of minimal complex I substrate (glutamate/malate, 5/2 µM), *Protocol 2*; progressively adding saturating concentrations of the following: 25 µM palmitoyl-carnitine + 1 mM malate, 2 mM glutamate, and 3 mM succinate.

There were no significant differences in H_2O_2 emission among the four groups in response to protocol 1 (Figure 7 A). However, high-fat plus exercise group (PCMGS: 8.002 ± 28.11 pmol/m/mg dry wt, and PCMGSG3P: 16.93 ± 21.33 pmol/m/mg dry wt) showed higher H_2O_2 emission than control group in response to protocol 2 (PCMGS: 18.32 ± 34.57 pmol/m/mg dry wt, PCMGSG3P: 31.39 ± 45.22 pmol/m/mg dry wt; $P<0.05$) (Figure 7 B).

Figure 2

Plasma glucose (A) and plasma insulin (B) concentration in response to oral glucose challenge.



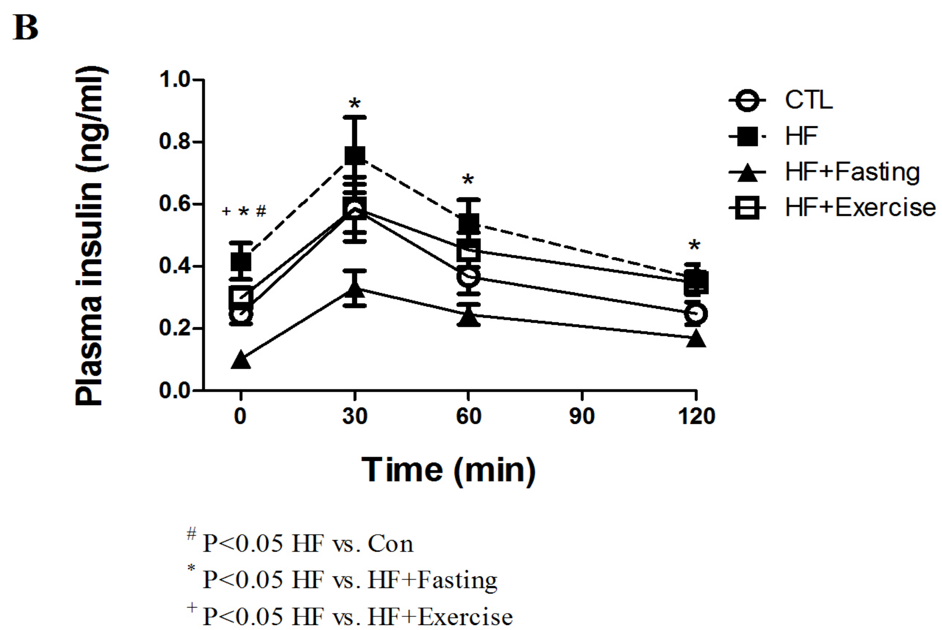
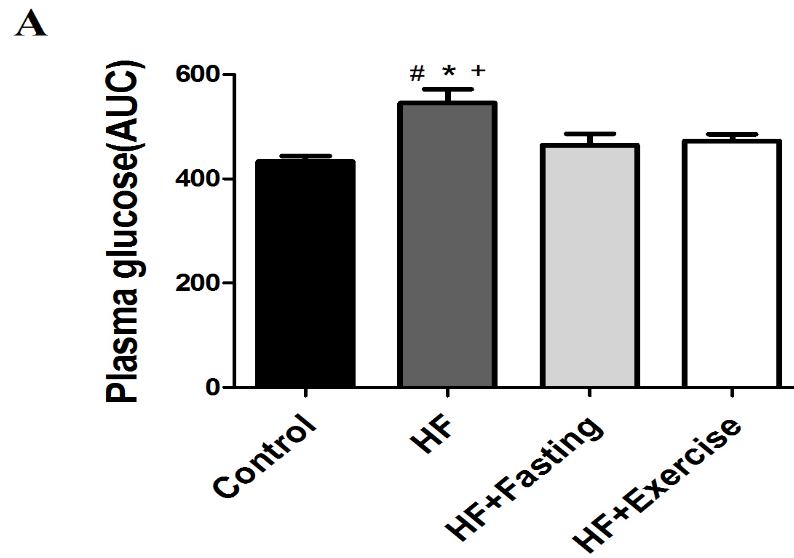
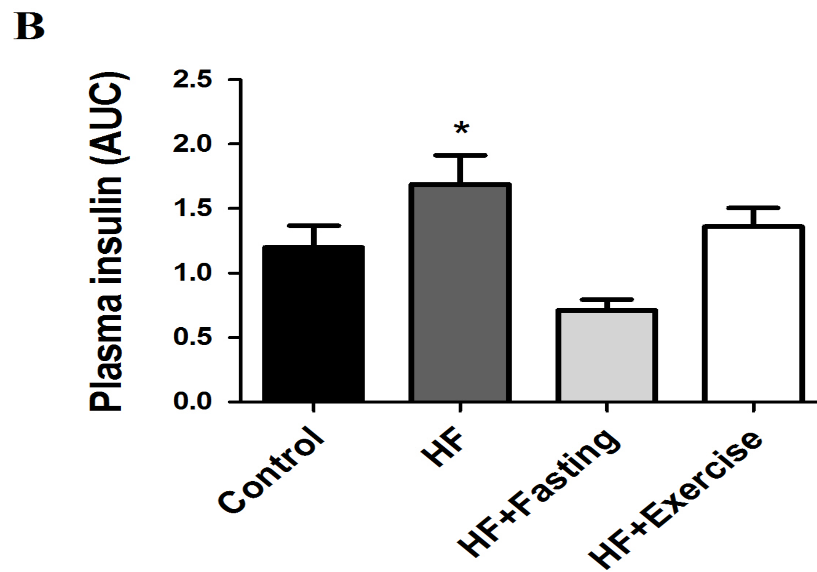


Figure 3

AUC (arbitrary units) for glucose (A) and for insulin (B) among groups.



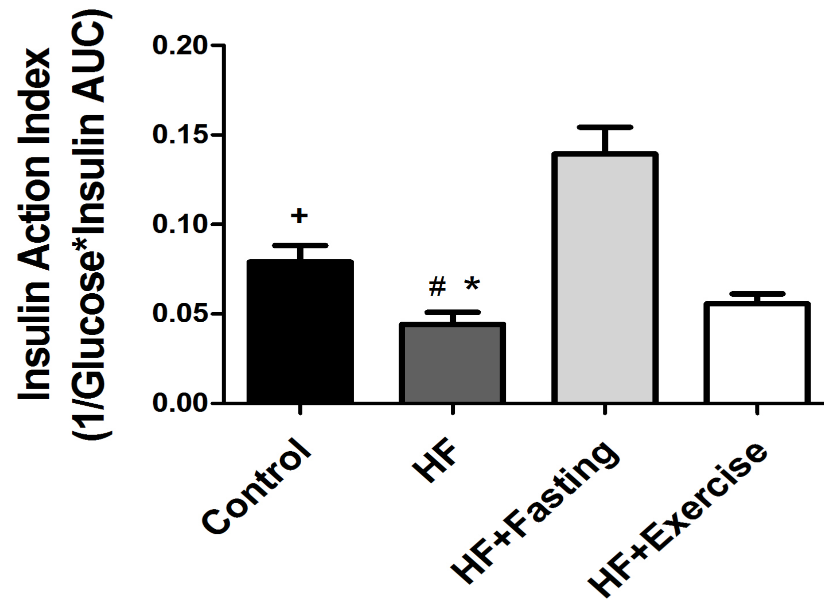
# P<0.05 HF vs. Con
* P<0.05 HF vs. HF+Fasting
+ P<0.05 HF vs. HF+Exercise



* P<0.05 HF vs. HF+Fasting

Figure 4

An index of insulin action among groups.



# P<0.05 HF vs. Con

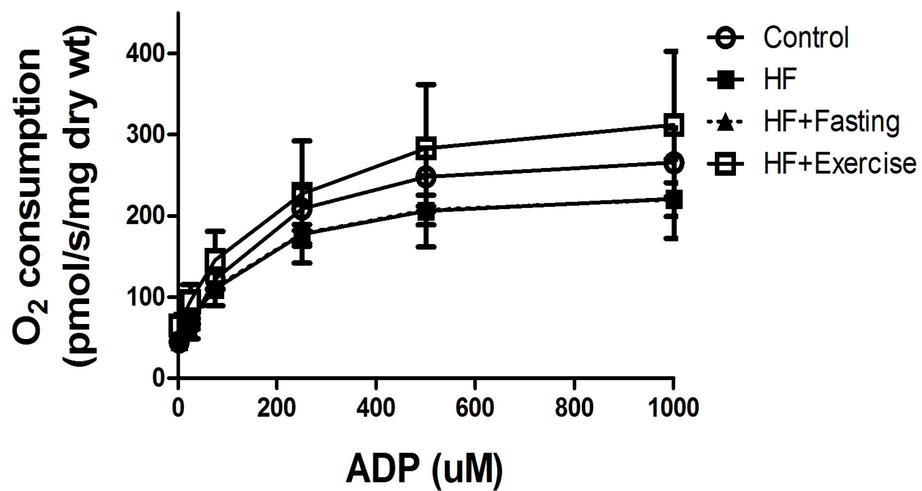
* P<0.05 HF vs. HF+Fasting

+P<0.05 Con vs. HF+Fasting

Figure 5

O_2 consumption rate determined from an ADP titration experiment (A) and O_2 consumption rate supported malate/glutamate, ADP, oligomycin, and FCCP (B).

A



B

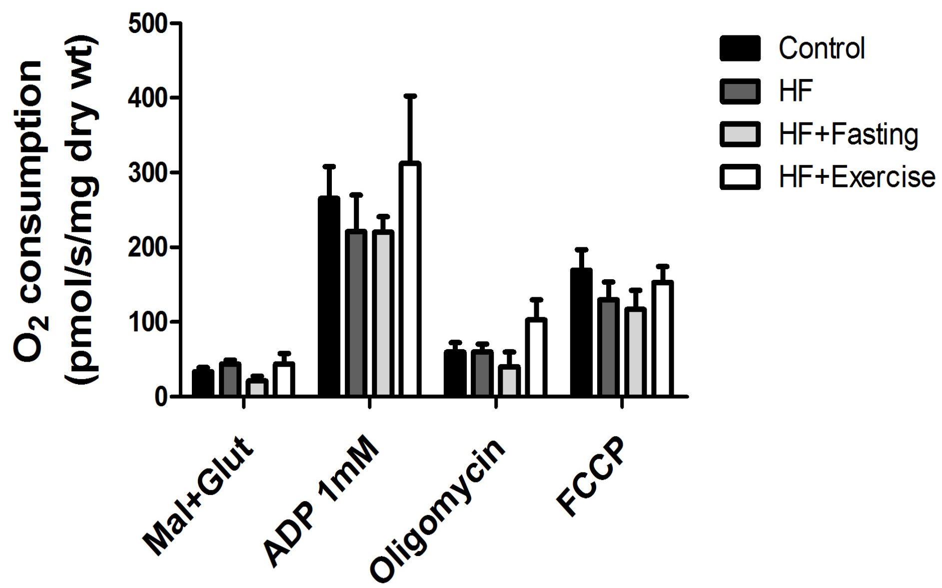
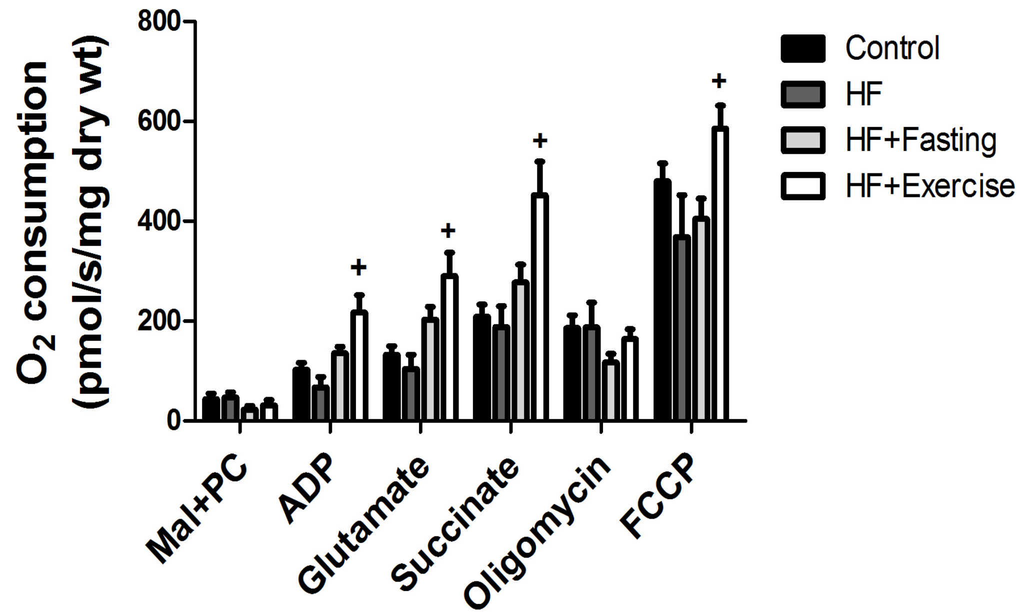


Figure 6

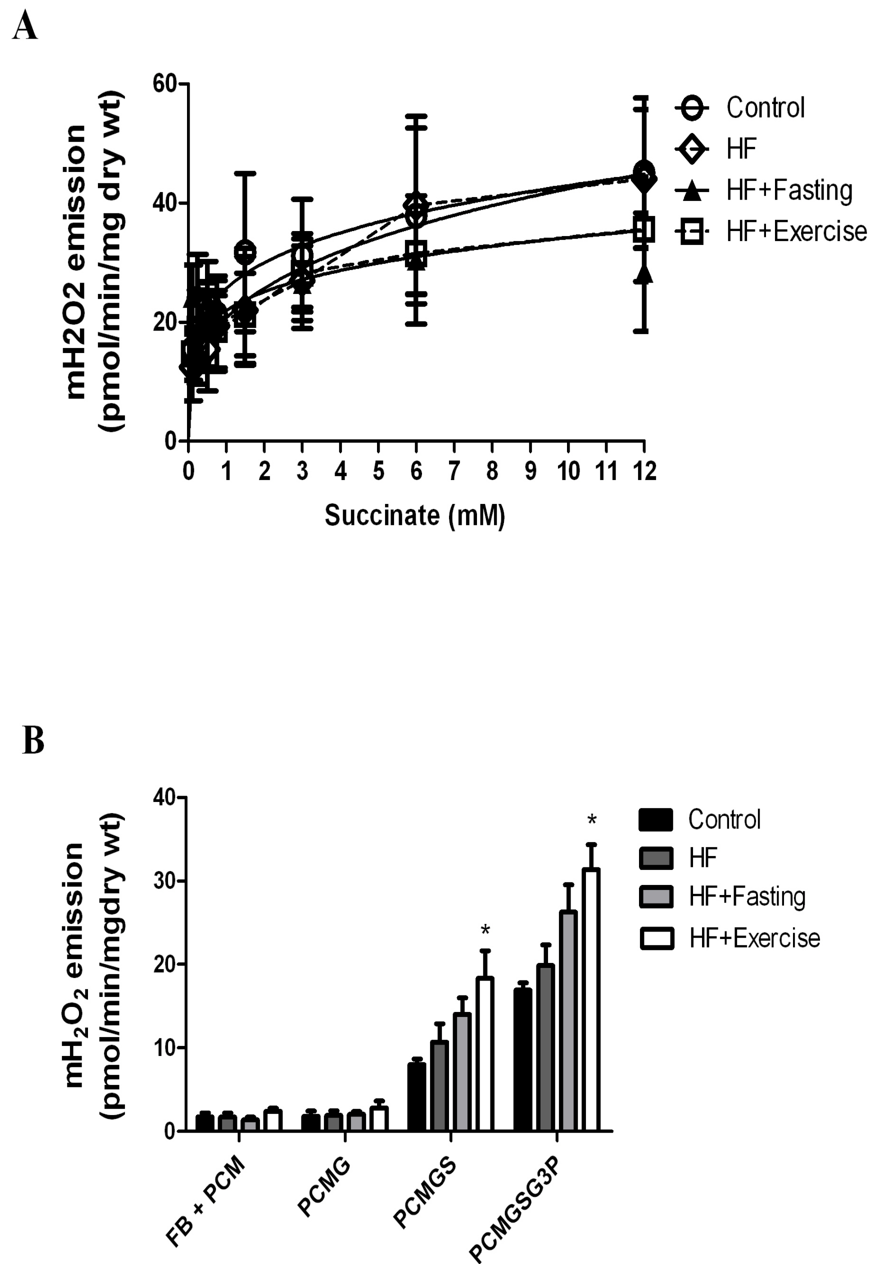
O_2 consumption from a palmitoylcarnitine plus malate experiment.



$^{+}$ $P<0.05$ HF vs. HF+Exercise

Figure 7

Succinate stimulated H_2O_2 emission (A) and palmitoylcarnitine plus malate stimulated H_2O_2 emission (B).



* P<0.05 Control vs.HF+Exercise

DISCUSSION

The major findings in the current study are that 1) an acute 16 h period of fasting dramatically improves whole body insulin action in rats fed a high-fat for 6 weeks, 2) a single low-intensity bout of treadmill exercise 16 h prior to testing does not improve insulin action but appears to enhance mitochondrial respiratory capacity in red gastrocnemius muscle and, 3) neither fasting nor exercise acutely alter the mitochondrial H_2O_2 emitting potential in red gastrocnemius muscle of rats consuming a high-fat diet.

Glucose, Insulin and Index of Insulin Action

We evaluated the role of acute fasting and acute exercise on indexes of whole body glucose metabolism in rats made insulin resistant by a high-fat diet. In particular, we were interested in determining whether a single acute period of fasting or a single bout of low-intensity exercise improves insulin action in rats consuming a high-fat diet and whether those improvements, if present, may be related to changes in mitochondrial function in skeletal muscle. In muscle, there is much evidence to suggest that lipid metabolite accumulation resulting from high dietary fat intake may play a causative role in the development of insulin resistance (Noland et al., 2007). A reduced capacity to oxidize lipids in skeletal muscle due to inherited and/or acquired mitochondrial dysfunction has also been implicated in the etiology of insulin resistance (Lowell and Shulman, 2005). Interestingly, Oakes et al. previously found that acute dietary lipid withdrawal (overnight fast or a single low-fat and high-glucose

meal) or a single exercise bout 18 h prior to testing were sufficient to restore insulin sensitivity in rats fed a high-fat diet for three weeks (Oakes et al., 1997). Although the mechanism(s) responsible for the reversal of insulin resistance were unclear, it is unlikely that such short-term physiological interventions were sufficient to improve mitochondrial functional capacity. However, data from our laboratory has recently indicated that high-fat diet induced loss of insulin sensitivity in muscle is linked to changes in mitochondrial H_2O_2 emission (Anderson et al., 2009). H_2O_2 emission is dynamic and governed by the balance between energy supply and demand within the mitochondria. Thus, we sought to determine whether short-term physiological interventions are sufficient to improve insulin action and whether such changes may be related to changes in mitochondrial function in skeletal muscle.

As in our previous study (Anderson et al., 2009), consuming a high-fat diet for six weeks induced insulin resistance. In response to the oral glucose challenge, glucose levels were significantly ($P<0.05$) higher at all time points in the high-fat diet group compared with controls as well as the high-fat plus fasting, and high-fat plus exercise groups. Plasma insulin levels of the high-fat plus fasting group showed significantly lower levels compared to high-fat diet group at 0, 30 min, 60 min, and 120 min after administration of glucose solution ($P<0.05$), suggesting an improved insulin sensitivity in high-fat plus fasting group. In order to provide a better indication of insulin sensitivity, we used an index of insulin action (Figure 4). Insulin action in high-fat plus fasting group was three-fold higher than high-fat diet group. Therefore, a single 16 h fast was sufficient to significantly improve insulin sensitivity in rats maintained on a high-fat diet. These data are consistent with the findings previously reported by Oakes et al. (Oakes et al., 1997).

In obesity, skeletal muscle has a reduced oxidative capacity, which is a metabolic characteristic associated with insulin resistance (Menshikova et al., 2007). In clinical investigations, exercise and/or caloric restriction are typically associated with improvements in insulin sensitivity and increased capacity for fat oxidation (Menshikova et al., 2007 and Bevilacqua et al., 2005). Although there are several studies using long-term exercise training as an intervention, very few studies have examined whether acute interventions that create a negative energy balance can reverse insulin resistance.

Oakes et al. studied the influence of a single bout of prior exercise on insulin resistance (Oakes et al., 1997). In this study, rats performed 2 h swimming exercise and insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamps the following morning, ~18 h after the exercise bout. Insulin-stimulated muscle glucose uptake was significantly increased indicating at least partial restoration of insulin sensitivity. Lima et al. also demonstrated that a single 2 h bout of swimming exercise improves insulin signaling in hepatic tissue of mice fed a high fat diet for two months (Lima et al., 2009). In response to an insulin injection, Akt phosphorylation as well as the expression of several transcription factors increased in the liver of mice that had exercised 8 h prior as compared with high-fat fed mice that did not exercise. Both the study by Oakes et al. and Lima et al. used 2 h swimming as the exercise stimulus, a mode of exercise that likely required considerably more effort than the low-intensity treadmill exercise employed in the present study (Oakes et al., 1997 and Lima et al., 2009). Although speculative, it is possible that 16 h of recovery prior to testing in the present study was a sufficient enough period of time for the metabolic surplus due of the high-fat diet to be re-established, thus negating the positive benefits of the

exercise. Further work will be required to better understand the interaction between cellular metabolic balance and insulin sensitivity in tissues.

O_2 Respiratory Function in Permeabilized Myofibers

One of the objectives of this study was to determine in rats consuming a high-fat diet whether acute physiological interventions such as fasting or low-intensity exercise alter mitochondrial function in skeletal muscle. Oxidative capacity in skeletal muscle is reduced in obese compared with lean, more physically active individuals and it has been suggested that this reduction in oxidative capacity may contribute to the accumulation of lipid within muscle fibers associated with obesity-induced insulin resistance (Menshikova et al., 2007). Oxidative capacity is assumed to be largely a function of mitochondrial content and thus it is unlikely that acute restoration of insulin sensitivity in response to acute interventions such as fasting or a single bout of exercise is mediated by an increase in oxidative capacity given the time required for mitochondrial biogenesis. Thus, other aspects of mitochondrial function must be at play if indeed mitochondrial function is linked to the control of insulin sensitivity.

ADP is considered to be one of the major triggers of oxidative energy production in skeletal muscle and the ADP sensitivity reflects the affinity of mitochondrial respiration for ADP (Walsh et al., 2001). In present study, no change in ADP sensitivity nor in maximal ADP-stimulated respiration was found among any of the groups during respiration supported by complex I substrates. To our knowledge, no previous studies have examined the potential impact of acute exercise on ADP-stimulated respiratory kinetics. However, several studies have showed that endurance

exercise training enhances maximal ADP-stimulated respiration (Walsh et al., 2001), although the impacts on the kinetics of ADP-stimulated respiration are less clear.

Walsh et al. studied the effect of endurance exercise training on oxidative function in permeabilized muscle fibers from humans (Walsh et al., 2001). Interestingly, in their study the sensitivity of the mitochondrion to ADP after endurance training decreased, consistent with the fact that ADP sensitivity is inversely related with mitochondrial oxidative capacity. In the present study, we hypothesized that changes in ADP sensitivity and/or maximal ADP-stimulated respiration may occur as a consequence of as yet unidentified regulatory changes to the respiratory system caused by high fat diet-induced shifts in the redox state of the mitochondria. A more oxidized redox environment has been shown to lead to glutathionylation and/or nitrosation of protein thiols of mitochondrial proteins (Dahm et al., 2006). However, our findings revealed no change in ADP sensitivity nor maximal ADP-stimulated respiration, at least during respiration with complex I substrates, and therefore do not support this hypothesis.

However, we also used another protocol that used multiple substrates featuring PCM, glutamate and succinate to more closely represent the *in vivo* state. In this protocol, respiration supported by fatty acids (palmitoylcarnitine plus malate with succinate, complex I + II substrates) showed significantly higher respiration capacity between the high-fat diet plus exercise versus the high-fat diet groups ($P<0.05$). This finding is therefore consistent with the hypothesis that exercise somehow alters the respiratory system, most likely at some point beyond complex I, that leads to an improvement in maximal flux capacity in response to ADP. Clearly, further work is

required to better establish and understand the potential regulatory mechanisms affecting mitochondrial respiratory function.

H_2O_2 Emission in Permeabilized Myofibers

We hypothesized that high-fat diet induces higher H_2O_2 emitting potential similar to our previous study that showed switching rats from a standard high-carbohydrate chow diet to 100% fat for 3 days or a 60% high-fat diet for 3 weeks induced a remarkable 3- to 4-fold increase in the maximal rate of mitochondrial H_2O_2 emission (Anderson et al., 2009). In our previous study, the increase in the potential to emit H_2O_2 in permeabilized fibers from rats consuming a high-fat diet suggested that the governance of electron leak from the respiratory system is subject to some type of allosteric regulation. Moreover, the increase in mitochondrial H_2O_2 emitting potential was prevented through the use of either pharmacological or transgenic mitochondrial-targeted antioxidants, providing evidence that elevated oxidant production and H_2O_2 emission in response to the dietary lipid overload is triggering allosteric regulation of the respiratory system. The implication is that components of the respiratory system are subject to redox regulation and that such regulation is impacted by the relative state of energy balance within the tissue. The dynamic nature of this regulation led us to hypothesize that the elevated H_2O_2 emitting potential induced by dietary lipid overload may be reversed by interventions such as fasting and/or exercise that acutely reestablish cellular metabolic balance. However, although the high-fat plus fasting group showed lower H_2O_2 emission tendency, there was no significant difference in

H_2O_2 emitting potential among groups. Further work is required to better understand the acute and chronic factors affecting H_2O_2 emission.

Summary

The result of this study suggested that the biological status of skeletal muscle fibers, including the degree of insulin sensitivity, is functionally linked to factors associated with metabolic balance. Remarkably, a single 16 h overnight fast is sufficient to restore insulin sensitivity in high fat diet-induced insulin resistant rats, providing evidence that insulin action in muscle is acutely sensitive to the metabolic state of cells. However, contrary to a previous study by Oakes et al., in the present study a single bout of low-intensity exercise on the day before testing failed to restore insulin action (Oakes et al., 1997). Interestingly, ADP-stimulated respiratory capacity was slightly but significantly higher in these animals, providing evidence of potential allosteric regulation of the respiratory system. Somewhat surprisingly however, neither fasting nor exercise altered the H_2O_2 emitting potential in permeabilized fibers, suggesting that further work is required to better understand the factors influencing mitochondrial function in relation to insulin sensitivity.

REFERENCES

Anderson, E. J., M. E. Lusting, et al. (2009). “Mitochondrial H_2O_2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans.” J Clin Invest **119**: 573-81.

Bayraktutan, U. (2002). “Free radicals, diabetes and endothelial dysfunction.” Diabetes Obes Metab **4**: 224-38.

Bergsten, P. (2000). “Pathophysiology of impaired pulsatile insulin release.” Diabetes/Metabolism Research and Reviews **16**(3): 179-91.

Bevilacqua, L., J. J. Ramsey, et al. (2005). “Long-term caloric restriction increases UCP3 content but decreases proton leak and reactive oxygen species production in rat skeletal muscle mitochondria.” Am J Physiol Endocrinol Metab **289**: E429-38.

Boushel, R., E. Gnaiger, et al. (2007). “Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle.” Diabetologia **50**: 790-6.

Bruce C. R., A. B. Thrush, et al. (2006). “Endurance training in obese humans improves glucose tolerance and mitochondrial fatty acid oxidation and alters muscle lipid content.” Am J Physiol Endocrinol Metab **291**: E99-107.

Bulow, J. and J. Madsen (1976). “Adipose tissue blood flow during prolonged, heavy exercise.” Pflugers Arch **363**:231-4.

Bulow, J. and J. Madsen (1981). “Influence of blood flow on fatty acid mobilization from lipolytically active tissue.” Pflugers Arch **390**: 169-74.

Coppey, L. J., J. S. Gellett, et al. (2003). “Preventing superoxide formation in epineurial arterioles of the sciatic nerve from diabetic rats restores endothelium dependent vasodilation.” Free Radic Res **37**: 33-40.

Dahm, C. C., K. Moore, et al. (2006). “Persistent S-nitrosation of complex 1 and other mitochondrial membrane proteins by S-nitrosothiols but not nitric oxide or peroxynitrite: implications for the interaction of nitric oxide with mitochondria.” J Biol Chem **281**(15): 10056-65.

Denton, R. and P. Randle (1967). “Concentrations of glycerides and phospholipids in rat heart and gastrocnemius muscles. Effect of alloxan-diabetes and perfusion.” Biochem J **104**(2): 416-22.

Du, X. L., D. Edelstein, et al. (2001). “Hyperglycemia inhibits endothelial nitric oxide synthase activity by posttranslational modification at the Akt site.” J Clin Invest **108**: 1341-8.

Frederico G. S., S. Watkins, et al. (2006). “Changes induced by physical activity and weight loss in the morphology of intermyofibrillar mitochondria in obese men and women.” J Clin Endocrinol Metab **91**: 3224-7.

Goodpaster, B. H., A. Katsiaras, et al. (2003). “Enhanced fat oxidation through physical activity is associated with improvements in insulin sensitivity in obesity.” Diabetes **52**: 2191-7.

Goodpaster, B. H. and D. Wolf (2004). “Skeletal muscle lipid accumulation in obesity, insulin resistance, and type 2 diabetes.” Pediatric Diabetes **5**: 219-26.

Goodpaster, B. H. and N. F. Brown (2005). “Skeletal muscle lipid and its association with insulin resistance: What is the role for exercise?” Exerc Sport Sci Rev **33**(3): 150-4.

Hedley, A. A., C. L. Ogden, et al. (2004). “Prevalence of overweight and obesity among US children, adolescents, and adults, 1999-2000.” JAMA **291**(23): 2847-50.

Hegarty, B. D., G. J. Cooney, et al. (2002). “Increased efficiency of fatty acid uptake contributes to lipid accumulation in skeletal muscle of high fat-fed insulin-resistant rats.” Diabetes **51**: 1477-84.

Houmard, J. A. (2007). “Do the mitochondria of obese individuals respond to exercise training?” J Appl Physiol **103**: 6-7.

Houmard, J. A. (2008). "Intramuscular lipid oxidation and obesity." Am J Physiol Regul Integr Comp Physiol **294**: R1111-6.

Horowitz, J. F. (2007). "Exercise-induced alterations in muscle lipid metabolism improve insulin sensitivity." Exerc Sport Sci Rev **35**(4): 192-6.

Huang, X., K-F, Eriksson, et al. (1999). "Insulin-regulated mitochondrial gene expression is associated with glucose flux in human skeletal muscle." Diabetes **48**: 1508-14.

Jackson, M. J. (2000). "Exercise and oxygen radical production by muscle. In Exercise and oxygen toxicity(ed. C. K. Sen, L. Packer & O. Hanninen)", pp. 57-68, 2nd edition. Amsterdam: Elsvier.

Jackson, M. J. (2005). "Reactive oxygen species and redox-regulation of skeletal muscle adaptations to exercise." Phil Trans R Soc B **360**: 2285-91.

Kelley, D. E., J. He, et al. (2002). "Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes." Diabetes **51**: 2944-50.

Kirkwood, S. P., F. Zurlo, et al. (1991) "Muscle mitochondrial morphology, body composition, and energy expenditure in sedentary individuals." Am J Physiol Endocrinol Metab **260**: E89-94.

Klein, S., E. J. Peters, et al. (1989). "Effect of short- and long-term beta-adrenergic blockade on lipolysis during fasting in humans." Am J Physiol **257**: E65-73.

Korshunov, S. S., V. P. Skulachev, et al. (1997). "High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria." FEBS Lett **416**: 15-8.

Kraegen, E. W. and G. J. Cooney (2008). "Free fatty acids and skeletal muscle insulin resistance." Curr Opin Lipidol **19**(3): 235-41.

Krogh, A. and J. Lindhard (1920). "The relative value of fat and carbohydrates as sources of muscular energy." Biochem J **14**: 290-363.

Lamson, D. W. and S. M. Plaza (2002). "Mitochondrial factors in the pathogenesis of diabetes: a hypothesis for treatment." Altern Med Rev **7**: 94-111.

Lima, A. F., E. R. Ropelle, et al. (2009). "Acute exercise reduces insulin resistance-induced TRB3 expression and amelioration of the hepatic production of glucose in the liver of diabetic mice." J Cell Physiol **221**: 92-7.

Lowell, B. B. and G. I. Shulman (2005). "Mitochondrial dysfunction and type 2 diabetes." Science **21**; **307**(5708): 384-7.

Maechler, P. and C. B. Wollheim (2001). "Mitochondrial function in normal and diabetic beta-cells." Nature **414**: 807-12.

McGarry, J.D. (2002). "Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes." Diabetes **51**: 7-18.

Menshikova, E. V., V. B. Ritov, et al. (2007). "Characteristics of skeletal muscle mitochondrial biogenesis induced by moderate-intensity exercise and weight loss in obesity." J Appl Physiol **103**(1): 21-7.

Mogensen, M. , K. Sahlin, et al. (2007) "Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes." Diabetes **56**(6): 1592-9.

Moro, C., S. Bajpeyi, et al. (2008). "Determinants of intramyocellular triglyceride turnover: Implications for insulin sensitivity." Am J Physiol Endocrinol Metab **294**: E203-13.

Nishikawa, T., D. Edelstein, et al. (2000). "Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage." Nature **404**: 787-90.

Noland, R. C., T. L. Woodlief, et al. (2007). "Peroxisomal-mitochondrial oxidation in a rodent model of obesity-associated insulin resistance." Am J Physiol Endocrinol Metab **293**: E986-1001.

Oakes, N. D., K. S. Bell, et al. (1997). "Diet-induced muscle insulin resistance in rats is ameliorated by acute dietary lipid withdrawal or a single bout of exercise." Diabetes **46**: 2022-8.

Pan D. A., S. Lillioja, et al. (1997). "Skeletal muscle triglyceride levels are inversely related to insulin action." Diabetes **46**: 983-8.

Patti M. E., A. J. Butte, et al. (2003). "Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1." Proc Natl Acad Sci USA **100**: 8466-71.

Petersen, K. F., D. Befroy, et al. (2003). "Mitochondrial dysfunction in the elderly: possible role in insulin resistance." Science **300**: 1140-2.

Petersen, K. F. and G. I. Shulman (2006). "Etiology of insulin resistance." Am J Med **119**: S10-6.

Puntschart, A., H. Claassen, et al. (1995). "mRNAs of enzymes involved in energy metabolism and mtDNA are increased in endurance-trained athletes." Am J Physiol Cell Physiol **269**: C619-25.

Ritov, V. B., E. V. Menshikova, et al. (2005). "Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes." Diabetes **54**: 8-14.

Ross, R., D. Dagnone, et al. (2000). "Reduction in obesity and related comorbid conditions after diet-induced weight loss or exercise-induced weight loss in men. A randomized, controlled trial." Ann Intern Med **133**: 92-103.

Saks, V. A., O. Kongas, et al. (2000) "Role of the creatine/phosphocreatine system in the regulation of mitochondrial respiration." Acta Physiol Scand **168**: 635-41.

Schafer, F. Q. and G. R. Buettner (2001). "Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple." Free Radic Biol Med **30**: 1191-212.

Schrauwen, P. (2007). "High-fat diet, muscular lipotoxicity and insulin resistance." Proc Nutrition Soc **66**: 33-41.

Sparks, L. M., H. Xie, et al. (2005). "A high-fat diet coordinately downregulates genes required for mitochondrial oxidative phosphorylation in skeletal muscle." Diabetes **54**: 1926-33.

Stone, J. R. and S. Yang (2006). "Hydrogen peroxide: a signaling messenger." Antioxid Redox Signal **8**: 243-70.

Stump, C. S., K. R. Short, et al. (2003). "Effect of insulin on human skeletal muscle mitochondrial ATP production, protein synthasis, and mRNA transcripts." Proc Natl Acad Sci USA **100**: 7996-8001.

Toledo, F. G., S. Watkins, et al. (2006). "Changes induced by physical activity and weight loss in the morphology of intermyofibrillar mitochondria in obese men and women." J Clin Endocrinol Metab **91**: 3224-7.

Tonkonogi, M., B. Walsh, et al. (2000). "Mitochondrial function and antioxidative defence in human muscle: effects of endurance training and oxidative stress." J Physiol(Lond) **528**: 379-88.

Turcotte, L. P., B. Kiens, et al. (1991). "Saturation kinetics of palmitate uptake in perfused skeletal muscle." FEBS Lett **279**: 327-9.

Turcotte, L. P., J. R. Swenberger, et al. (1999). "Training-induced elevation in FABPpm is associated with increased palmitate use in contracting muscle." J Appl Physiol **87**: 285-93.

Walsh, B., M. Tonkonogi, et al. (2001). "Effect of endurance training on oxidative and antioxidative function in human permeabilized muscle fibres." Eur J Physiol **442**: 420-5.

Wautier, M. P., O. Chappey, et al. (2001). "Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE." Am J Physiol Endocrinol Metab **280**: E685-94.

APPENDIX

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

December 5, 2006

Darrell Neufer, Ph.D.
Department of Physiology/EXSS
Brody 6W-39
ECU Brody School of Medicine

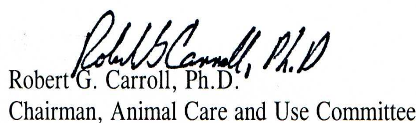
Dear Dr. Neufer:

Your Animal Use Protocol entitled, "Mitochondrial Bioenergetics and Metabolic Disease - Rats," (AUP #Q238) was reviewed by this institution's Animal Care and Use Committee on 12/5/06. The following action was taken by the Committee:

"Approved as submitted"

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,



Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

East Carolina University is a constituent institution of the University of North Carolina. An Equal Opportunity/Affirmative Action Employer.