SKELETAL MUSCLE METABOLIC FLEXIBILITY IMPAIRMENTS IN RESPONSE TO LIPID WITH OBESITY: EFFECT OF EXERCISE TRAINING

by

Gina Battaglia

August, 2012

Director of Dissertation: Joseph A. Houmard, Ph.D

Major Department: Department of Kinesiology

Obese individuals exhibit skeletal muscle metabolic inflexibility by failing to increase fat oxidation and genes linked with mitochondrial biogenesis in response to a high-fat diet (HFD) and lipid incubation in cell culture. Exercise training can increase skeletal muscle fatty acid oxidation (FAO) and mitochondrial content in the skeletal muscle of the obese, although whether it can normalize the response to excess lipid is unclear. The purpose of this dissertation was to determine whether metabolic flexibility in response to lipid could be rescued by short-term aerobic exercise training (study 1) and chemically uncoupling primary human skeletal muscle cells (HSkMC) to increase cell energy expenditure (study 2) in obese skeletal muscle. The hypotheses were that 1) obese individuals would exhibit skeletal muscle metabolic inflexibility in response to a 3d eucaloric high-fat diet (HFD; 70% total calories) in study 1, a defect that would persist in response to 24h lipid incubation in HSkMC (study 2); and 2) 10 consecutive days of aerobic exercise training (study 1), and mild uncoupling *in vitro* (study 2) would normalize the response to excess lipid in the skeletal muscle of obese individuals. Unlike the lean, obese individuals did not increase skeletal muscle FAO in response to a 3d HFD while

sedentary (study 1) and did not increase protein content of Complex I, Complex III, and COX IV in response to 24h lipid incubation in HSkMC (study 2), supporting our hypothesis of lipid-induced metabolic inflexibility in obese skeletal muscle. Exercise training combined with a HFD produced a similar increase in FAO and citrate synthase (CS) activity in lean and obese individuals (study 1), indicating that HFD-induce metabolic flexibility was restored with exercise training regardless of obesity status. Incubation with the chemical uncoupler FCCP and lipid exhibited a treatment effect for Complex I and Complex III, although the latter appeared to be driven by the robust lean response, and increased COX IV content in the lean only. Therefore, lipid-induced metabolic inflexibility is persistent in cell culture, and increasing energy expenditure *in vitro* does not fully restore the response to lipid in a manner similar to *in vivo* exercise training.

SKELETAL MUSCLE METABOLIC FLEXIBILITY IMPAIRMENTS IN RESPONSE TO LIPID WITH OBESITY: EFFECT OF EXERCISE TRAINING

A Dissertation

Presented to the Faculty of the Department of Kinesiology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy

by

Gina Battaglia

August, 2012

SKELETAL MUSCLE METABOLIC FLEXIBILITY IMPAIRMENTS IN RESPONSE TO LIPID WITH OBESITY: EFFECT OF EXERCISE TRAINING

By

Gina Battaglia

APPROVED BY:

DIRECTOR OF DISSERTATION/THESIS:_____Joseph A. Houmard, Ph.D

COMMITTEE MEMBER: _____David A. Brown, Ph.D

COMMITTEE MEMBER: _____P. Darrell Neufer, Ph.D

COMMITTEE MEMBER: _____Jacques Robidoux, Ph.D

COMMITTEE MEMBER: _____Carol A. Witczak, Ph.D

CHAIR OF THE DEPARTMENT OF KINESIOLOGY_____Stacey R. Altman, Ph.D

DEAN OF THE GRADUATE SCHOOL_____ Paul J. Gemperline, PhD

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

ASM: acid soluble metabolites

ATP: adenosine triphosphate

BCA: bicinchoninic acid

BMI: body mass index

BSA: bovine serum albumin

CaMK: Ca^{2+}/calmodulin-dependent protein kinase

COX: cytochrome c oxidase

CPT-1: carnitine palmitoyl transferase-1

CS: citrate synthase

DMEM: Dulbecco Modified Eagle's Medium

DNA: deoxyribonucleic acid

DNP: 2,4 dinitrophenol

ECAR: extracellular acidification rate

$FADH_2$: reduced flavin adenine dinucleotide

FAO: fatty acid oxidation

FAT/CD36: fatty acid translocase

FCCP: carbonyl cyanide p-(triflurometoxy) phenyl hydrazone

HAD: beta-hydroxyacyl coenzyme A dehydrogenase

HFD: high-fat diet

HOMA-IR: homeostasis model of assessment-insulin resistance

HSkMC: primary human skeletal muscle cells raised in culture

MAPK: p38 mitogen-activated protein kinase

mRNA: messenger ribonucleic acid

mtDNA: mitochondrial DNA

NADH: reduced nicotinamide adenine dinucleotide

OCR: oxygen consumption rate

OXPHOS: oxidative phosphorylation

PDH: pyruvate dehydrogenase complex

PDK4: pyruvate dehydrogenase kinase 4

PGC-1: peroxisome proliferator-activated receptor γ co-activator

PPAR: peroxisome proliferator-activated receptor

RER: respiratory exchange ratio

SEM: standard error of the mean

VO_2peak: peak rate of oxygen consumption

CHAPTER 1: LITERATURE REVIEW

Obesity and Metabolic Flexibility

Obesity has become the most prevalent disorder of the 21st century, affecting over one-third of the United States population (~72 million individuals) and contributing to at least 112,000 preventable deaths per year as well as diabetes, heart disease, and disability (6). The skeletal muscle of lean, healthy individuals has the capacity to shift between carbohydrate and fat utilization depending on fuel availability, a phenomenon known as metabolic flexibility (11). One of the critical aspects of obesity which may contribute to an increased incidence for disease is a pronounced impairment in metabolic flexibility, particularly in skeletal muscle.

This dissertation focused on the effects of increased lipid presence on lipid oxidation in respect to metabolic flexibility and obesity. In lean individuals, a high-fat diet has been shown to increase whole-body lipid oxidation (20), as well as transcription of genes involved in fatty acid transport (FAT/CD36), oxidation (HAD) (3), and substrate switching (PDK4) (15) in skeletal muscle. This indicates that lean individuals are able to adjust to lipid overload and increase oxidation, indicative of metabolic flexibility. However, there is evidence that this increase in lipid oxidation in response to a high-fat diet does not occur in obese individuals (i.e. they are metabolically inflexible) (9). This mismatch between lipid availability and oxidation ultimately leads to increased presence of triglycerides and fatty acid intermediates in skeletal muscle, which has been implicated in the development of insulin resistance and type 2 diabetes (14). A reduced capacity to oxidize fat has also been linked to weight gain in humans (16), and skeletal muscle cells cultured from diabetic individuals have reduced lipid oxidation when compared to nondiabetic controls (7), suggesting that reduced lipid oxidation is a persistent trait. Our recent

data has shown that 5 d of a 60% fat diet increases skeletal muscle palmitate oxidation and expression of genes involved in lipid oxidation in lean, but not obese men and women (2). The molecular mechanism behind this repressed response to lipid in skeletal muscle with obesity is unclear as well as the impact of an intervention such as exercise training.

Mitochondrial Content, Obesity, and Lipid Oxidation

Since complete lipid oxidation exclusively involves mitochondria, studying mitochondrial content and/or function is critical in determining the capacity to increase lipid oxidation. Studies have shown that obese individuals have reduced activities of β-hydroxyacyl CoA dehydrogenase (HAD) and citrate synthase (CS), as well as reduced cytochrome c oxidase IV (COX IV) content (8,10,12,19) compared to their lean counterparts which suggests that deficiencies with obesity may simply be a function of reduced mitochondrial content. In support, a study showed that obese individuals had reduced fatty acid oxidation (FAO) in skeletal muscle homogenates, but FAO did not differ after adjusting for mitochondrial mass, again implicating reduced mitochondrial content (8). Recent research from our laboratory showed the decreased FAO in obese human myotubes was abolished after normalization to mitochondrial DNA (mtDNA) and COX IV content, respectively (4). This finding in cell culture suggests that the reduction in mitochondrial content is imprinted in the genetic program of primary myotubes and may play a key role in determining the response to exogenous lipid.

Can Exercise Improve Metabolic Flexibility in Skeletal Muscle?

Aerobic exercise training increases oxidative enzyme activities, and even short-term exercise training (9 days) has been shown to increase the transcription of genes involved in lipid transport in lean individuals (23). Additionally, exercise is an intervention that has had

promising results in increasing the capacity for lipid oxidation in obese individuals. For example, gastric bypass-induced weight loss did not increase lipid oxidation in obese individuals, but ten consecutive days of training normalized lipid oxidation to the level of lean individuals who underwent the same exercise training (1). Additionally, mitochondrial content and electron transport chain activity increased after a 16-week diet and exercise program, whereas weight loss alone actually decreased mitochondrial size, strongly suggesting that exercise is a potent stimulus for increasing mitochondrial content and/or function in overweight/obese individuals (22). Whether metabolic flexibility can be restored with exercise training in obese individuals has not been investigated; however, our finding that 10 days of exercise restored oxidative capacity, as well as research demonstrating increased mitochondrial content with exercise training, strongly suggests that it can.

Multiple signaling pathways associated with exercise, namely Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), AMP-activated protein kinase (AMPK), and p38 mitogen-activated protein kinase (MAPK), likely stimulate mitochondrial biogenesis (13). However, the convergence of these pathways and how they directly or indirectly control mitochondrial biogenesis is complex. Furthermore, manipulation of kinase activity or gene expression *in vitro* (i.e. genetic disruption of AMPK) may lead to non-physiological compensatory mechanisms created by the cell in an attempt to maintain homeostasis. Therefore, an *in vitro* exercise mimetic that increases overall metabolic activity within the cell may be optimal for studying the effects of metabolic pathways activated by contractile activity. In this respect, low concentrations of chemical uncouplers (FCCP, 2,4-dinitrophenol) have been used to study energy homeostasis in 10T ½ fibroblasts (17), intramyocellular lipid oxidation in human myotubes (5), and triglyceride (TG) synthesis in 3T3-L1 adipocytes (18, 21). Chemical

uncouplers disrupt the proton gradient (the driving force behind ATP production by the electron transport chain) across the inner mitochondrial membrane, which causes protons to leak back into the mitochondrial matrix. The dissipation of this proton gradient leads to increased cell respiration and substrate utilization in an attempt to maintain intracellular ATP, which triggers the cell to compensate for this temporary energy depletion via intracellular signaling. Rohas et al demonstrated that peroxisome proliferator-activated receptor γ co-activator (PGC-1), oxidative phosphorylation (OXPHOS) gene expression, and mitochondrial density increased after 16 h of mild uncoupling in fibroblasts. We utilized this property to determine if pharmacological uncoupling alone would increase mitochondrial content and cell respiration and restore the metabolic flexibility to lipid exposure in the skeletal muscle of obese individuals

Previous research strongly suggests that the skeletal muscle of obese individuals is metabolically inflexible in response to high lipid loads in terms of increasing lipid oxidation, a defect that persists in cell culture. Short-term exercise training has been shown to increase skeletal muscle lipid oxidation, but whether it can improve metabolic flexibility in response to lipid and if the increase in energy demand is the primary mechanism involved in these positive adaptations is unknown. The purpose of this dissertation is 1) determine whether the metabolic inflexibility in response to lipid in obese individuals can be rescued by short-term exercise training and 2) if increasing energy demand by chemical uncoupling is the primary mechanism involved with the positive changes seen with exercise.

References

1. **Berggren JR, Boyle KE, Chapman WH, and Houmard JA**. Skeletal muscle lipid oxidation and obesity: influence of weight loss and exercise. *Am J Physiol Endocrinol Metab* 294: E726-732, 2008.
2. **Boyle KE, Canham JP, Consitt LA, Zheng D, Koves TR, Gavin TP, Holbert D, Neufer PD, Ilkayeva O, Muoio DM, and Houmard JA**. A high-fat diet elicits differential responses in genes coordinating oxidative metabolism in skeletal muscle of lean and obese individuals. *The Journal of clinical endocrinology and metabolism* 96: 775-781, 2011.
3. **Cameron-Smith D, Burke LM, Angus DJ, Tunstall RJ, Cox GR, Bonen A, Hawley JA, and Hargreaves M**. A short-term, high-fat diet up-regulates lipid metabolism and gene expression in human skeletal muscle. *Am J Clin Nutr* 77: 313-318, 2003.
4. **Consitt LA, Bell JA, Koves TR, Muoio DM, Hulver MW, Haynie KR, Dohm GL, and Houmard JA**. Peroxisome proliferator-activated receptor-gamma coactivator-1alpha overexpression increases lipid oxidation in myocytes from extremely obese individuals. *Diabetes* 59: 1407-1415.
5. **Corpeleijn E, Hessvik NP, Bakke SS, Levin K, Blaak EE, Thoresen GH, Gaster M, and Rustan AC**. Oxidation of intramyocellular lipids is dependent on mitochondrial function and the availability of extracellular fatty acids. *Am J Physiol Endocrinol Metab* 299: E14-22.
6. **Flegal KM, Graubard BI, Williamson DF, and Gail MH**. Excess deaths associated with underweight, overweight, and obesity. *Jama* 293: 1861-1867, 2005.
7. **Gaster M, Rustan AC, Aas V, and Beck-Nielsen H**. Reduced lipid oxidation in skeletal muscle from type 2 diabetic subjects may be of genetic origin: evidence from cultured myotubes. *Diabetes* 53: 542-548, 2004.
8. **Holloway GP, Thrush AB, Heigenhauser GJ, Tandon NN, Dyck DJ, Bonen A, and Spriet LL**. Skeletal muscle mitochondrial FAT/CD36 content and palmitate oxidation are not decreased in obese women. *Am J Physiol Endocrinol Metab* 292: E1782-1789, 2007.
9. **Kelley DE, Goodpaster B, Wing RR, and Simoneau JA**. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* 277: E1130-1141, 1999.
10. **Kelley DE, He J, Menshikova EV, and Ritov VB**. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* 51: 2944-2950, 2002.
11. **Kelley DE, and Mandarino LJ**. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes* 49: 677-683, 2000.
12. **Kim JY, Hickner RC, Cortright RL, Dohm GL, and Houmard JA**. Lipid oxidation is reduced in obese human skeletal muscle. *Am J Physiol Endocrinol Metab* 279: E1039-1044, 2000.
13. **Lira VA, Brown DL, Lira AK, Kavazis AN, Soltow QA, Zeanah EH, and Criswell DS**. Nitric Oxide and AMPK cooperatively regulate PGC-1{alpha} in skeletal muscle cells. *J Physiol*.
14. **Pagel-Langenickel I, Bao J, Pang L, and Sack MN**. The role of mitochondria in the pathophysiology of skeletal muscle insulin resistance. *Endocr Rev* 31: 25-51.
15. **Peters SJ, Harris RA, Wu P, Pehleman TL, Heigenhauser GJ, and Spriet LL**. Human skeletal muscle PDH kinase activity and isoform expression during a 3-day high-fat/low-carbohydrate diet. *Am J Physiol Endocrinol Metab* 281: E1151-1158, 2001.

16. **Ravussin E**. Metabolic differences and the development of obesity. *Metabolism: clinical and experimental* 44: 12-14, 1995.
17. **Rohas LM, St-Pierre J, Uldry M, Jager S, Handschin C, and Spiegelman BM**. A fundamental system of cellular energy homeostasis regulated by PGC-1alpha. *Proc Natl Acad Sci U S A* 104: 7933-7938, 2007.
18. **Si Y, Shi H, and Lee K**. Metabolic flux analysis of mitochondrial uncoupling in 3T3-L1 adipocytes. *PLoS One* 4: e7000, 2009.
19. **Simoneau JA, Veerkamp JH, Turcotte LP, and Kelley DE**. Markers of capacity to utilize fatty acids in human skeletal muscle: relation to insulin resistance and obesity and effects of weight loss. *Faseb J* 13: 2051-2060, 1999.
20. **Smith SR, de Jonge L, Zachwieja JJ, Roy H, Nguyen T, Rood J, Windhauser M, Volaufova J, and Bray GA**. Concurrent physical activity increases fat oxidation during the shift to a high-fat diet. *Am J Clin Nutr* 72: 131-138, 2000.
21. **Tejerina S, De Pauw A, Vankoningsloo S, Houbion A, Renard P, De Longueville F, Raes M, and Arnould T**. Mild mitochondrial uncoupling induces 3T3-L1 adipocyte de-differentiation by a PPARgamma-independent mechanism, whereas TNFalpha-induced de-differentiation is PPARgamma dependent. *J Cell Sci* 122: 145-155, 2009.
22. **Toledo FG, Menshikova EV, Azuma K, Radikova Z, Kelley CA, Ritov VB, and Kelley DE**. Mitochondrial capacity in skeletal muscle is not stimulated by weight loss despite increases in insulin action and decreases in intramyocellular lipid content. *Diabetes* 57: 987-994, 2008.
23. **Tunstall RJ, Mehan KA, Wadley GD, Collier GR, Bonen A, Hargreaves M, and Cameron-Smith D**. Exercise training increases lipid metabolism gene expression in human skeletal muscle. *Am J Physiol Endocrinol Metab* 283: E66-72, 2002.

CHAPTER 2: SHORT-TERM EXERCISE TRAINING INCREASES HIGH-FAT DIET-INDUCED LIPID OXIDATION IN LEAN AND OBESE INDIVIDUALS

Abstract

Obese individuals fail to increase expression of lipid oxidation genes in response to a high-fat diet. Short-term exercise increases skeletal muscle lipid oxidation in obese individuals, although whether it can improve high-fat diet-induced lipid oxidation is unclear. The purpose of this study was to compare skeletal muscle lipid oxidation response to a 3-day high-fat diet (HFD) and 10 consecutive days of aerobic exercise training in sedentary lean and obese individuals. Twelve lean (age 21.8±1.1y; BMI 22.6±0.7 kg/m^2) and 10 obese (age 22.4±0.8y; BMI 33.7±0.7 kg/m^2) males consumed a HFD (70% of calories) for 3d. Ten consecutive days of aerobic exercise (1h/d, 70% VO_2peak) on a cycle ergometer was performed, and the diet was repeated during days 8-10 of exercise training. Fasting muscle biopsies were taken before and after each HFD and fatty acid oxidation (FAO) measured with end-labeled [1-^{14}C] palmitate. Indices of mitochondrial content were determined using Western blots and enzyme activity assays. Lean subjects increased FAO (mean ±SEM 27.3±7.4%, p=0.03) in response to a 3-day HFD, significantly more than their obese counterparts (1.0±7.9%). A HFD and exercise increased FAO by 64.0±32.8% in leans and by 70.4±34.8% in obese, compared to pre-diet, pre-exercise values. Citrate synthase (CS) and beta-hydroxyacyl coenzyme A dehydrogenase (HAD) activities followed a similar pattern, with overall treatment effects of 7d exercise and 10d exercise plus 3d HFD. Short-term exercise training rescued the FAO response to a HFD in obese individuals. Further research should investigate mechanisms responsible for improvement in HFD-induced skeletal muscle FAO. Keywords: skeletal muscle, fat oxidation, mitochondria, physical activity

Introduction

The prevalence of obesity has been increasing rapidly and is strongly associated with the development of insulin resistance and type 2 diabetes (11). An important indicator of metabolic health is metabolic flexibility, which is the ability to adjust substrate utilization based on substrate availability (18). For example in lean individuals, fatty acid oxidation at the whole-body level increases rapidly in response to a high fat diet; to the contrary, obese individuals display an impaired capacity to increase lipid oxidation with increased dietary lipid (1, 13, 17, 26). This inability to increase lipid oxidation when lipid presence is elevated creates a condition of positive fat balance resulting in lipid storage (34), which in skeletal muscle may lead to ectopic lipid accumulation (26), lipid peroxidation (28), and excessive increases in lipid intermediates such as diacylglycerol and ceramide resulting in intracellular lipotoxicity (21).

Skeletal muscle is a major contributor to whole-body lipid utilization and thus plays a role in relation to metabolic flexibility. In lean individuals a high-fat diet increased the transcription of genes involved in fatty acid transport and oxidation (3, 6, 23) in skeletal muscle. However, obese individuals did not increase the transcription of genes that control skeletal muscle lipid metabolism as compared to their lean counterparts in response to a 5d high fat diet (3), again demonstrating that inflexibility persists in skeletal muscle with obesity.

Endurance-oriented exercise training increases lipid oxidation in skeletal muscle in both lean and obese individuals. An increase in mitochondrial content has been reported with as little as 7 days of aerobic training (31). Furthermore, our studies have shown that formerly severely obese (BMI>40 kg/m^2) individuals who lost weight via gastric bypass surgery increased skeletal muscle lipid oxidation to the level of an exercise-trained lean individual with 10 days of aerobic

exercise (2). This suggests that obese individuals are capable of responding to exercise in terms of increasing the capacity for lipid oxidation in skeletal muscle. However, it is not evident if the capacity for metabolic flexibility in response to ingested lipid is also restored. The purposes of the present study were to 1) specifically determine if the skeletal muscle of obese individuals is metabolically inflexible in response to a short-term high-fat diet in terms of appropriately increasing lipid oxidation; and 2) to determine if exercise training can correct any impairment in metabolic flexibility evident with obesity.

Methods

Subjects. Twelve lean (BMI≤25 kg/m^2) and 10 obese (BMI≥30 kg/m^2) male subjects ages 18-30y volunteered to participate. Subjects were not involved in an aerobic training program, as determined by a physical activity questionnaire and verbal questioning, and were asked to not change their physical activity patterns throughout the duration of the study. Participants filled out a medical history to confirm they were free from disease, did not smoke, and were not taking any medications known to influence carbohydrate or lipid metabolism. Subjects were weight-stable (±2 kg over the past 3 months) and nonsmokers. The experimental procedure and associated risks were explained in written and oral format, and informed consent obtained. The study was approved by the East Carolina Policy and Review Committee on Human Research and was in accordance with the Declaration of Helsinki.

Study design. Each participant consumed a high-fat diet for 3 days while sedentary. After a 2-3 week washout period, subjects exercised for 10 consecutive days and consumed the high-fat diet at days 8-10 of exercise training (Fig. 2.1). Skeletal muscle biopsies were obtained from the vastus lateralis after a 12h overnight fast on the morning that the high fat diet was initiated and the morning after the 3 days of the diet.

Diet. The high-fat diet consisted of approximately 70 percent fat, 15 percent carbohydrate, and 15 percent protein. Energy content for each individual was determined from the Harris-Benedict equation (15). Food was provided and subjects logged their intake. The day prior to commencement of each of the high-fat diets, subjects were provided an isocaloric diet consisting of approximately 25 percent fat, 15 percent protein, and 60 percent carbohydrates. All diets and

food logs were analyzed by Nutritionist Pro Nutrition Analyst Software (Axxya Systems LLC; Stafford, TX) to ensure correct macronutrient composition.

Exercise training. An incremental maximal exercise test on an electronically braked cycle ergometer was performed to determine VO_2peak during the screening process. Participants then exercised 60 min/d at 70% VO_2peak for 10 consecutive days. All training was supervised and performed in the laboratory setting; heart rate was monitored throughout each training session and VO_2 measurements were taken periodically to ensure proper workload. Net calories burned during exercise training were estimated using the VO_2 measurements and the resulting energy added to the diets provided during days 8-10 of exercise. Exercise was performed 14-18h before the muscle biopsies on day 7 and day 10.

Muscle analyses. Fatty acid oxidation (FAO) was measured as described previously (2, 16, 19). Briefly, 50-60 mg of muscle tissue was collected in 200 µl of a buffer containing 250 mM sucrose, 1mM EDTA, 10mM Tris-HCl, and 2 mM ATP, pH 7.4. Samples were minced with scissors to remove excess fat and connective tissue and diluted 20-fold with additional buffer. Tissue was placed on ice and homogenized with a Teflon pestle for 30s. Forty microliters of homogenate was added to the top well of a sealed, modified 48-well plate that contained a channel connecting to the adjacent trap well, which allowed for the passage of CO_2 liberated by the complete oxidation of [1-^{14}C] palmitate. The bottom trap well contained 1 N sodium hydroxide to collect the $^{14}CO_2$ given off by the oxidation procedure. To initiate the reaction, 160 µl of a reaction buffer composed of the following was added to the top wells: 0.2mM palmitate ([1-^{14}C]palmitate at 0.5 µCi/ml), 100 mM sucrose, 10 mM Tris-HCl, 5mM potassium phosphate, 80 mM potassium chloride, 1mM magnesium chloride, 0.1 mM malate, 2 mM ATP, 1mM

dithiothreitol, 0.2 mM EDTA, 1 mM L-carnitine, 0.5 mM coenzyme A, and 0.5% fatty acid-free bovine serum albumin, pH 7.4. The samples were incubated in a 37°C water bath for 30 min, at which point 100 µl of 70% perchloric acid was added to terminate the reaction. The plate was placed on a shaker for 1h to ensure complete transfer of CO_2 into the bottom well. Label incorporation into $^{14}CO_2$ was determined by scintillation counting using 4 ml of Uniscient BD (National Diagnostics, Atlanta, GA). Incomplete oxidative products (acid-soluble metabolites, ASM) remaining in the top well were measured as described previously (19).

A 10-15 mg piece of muscle was homogenized at 4°C using a Bullet Blender (Next Advance, Averill Park, NY) in a lysis buffer containing 50 mM HEPES, 12 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 1% Triton X-100, and 0.1% SDS and supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Samples were rotated end-over-end on a rotating wheel for 1h at 4°C and centrifuged at 21,000g for 20 min at 10°C. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). Five micrograms of protein were separated by SDS-PAGE and electrotransferred to polyvinylidene flouride (PVDF) membranes (Millipore, Billerica, MA) and probed overnight for cytochrome c oxidase IV (1:1000; Cell Signaling, Beverly, MA) and with a cocktail containing antibodies against the following proteins (1:1000): Complex I subunit NDUFB8, Complex II subunit 30kDa, Complex III subunit Core 2, Complex IV subunit II, and ATP synthase subunit alpha (Mitosciences, Eugene, OR). Membranes were incubated for 1h at room temperature with the corresponding secondary antibody and the immunoreactive proteins were detected using enhanced chemiluminescence (ChemiDoc XRS+ Imaging System, BioRad Laboratories, Inc., Hercules, CA). Samples were normalized to a crude muscle homogenate sample on each gel to normalize for blotting efficiency across gels.

A 10-15 mg piece of muscle was diluted 20-fold in a buffer containing 100 mM KH_2PO_4 and 0.05% bovine serum albumin and homogenized at 4°C using a Bullet Blender. Homogenates went through four freeze-thaw cycles before experimentation. This homogenate was used for determining citrate synthase (CS) and beta-hydroxyacyl coenzyme A dehydrogenase (HAD) activity. Protein content was measured using the BCA assay. Citrate synthase activity was assessed with reagents provided in a kit (Sigma CS0720), which used a colorimetric reaction to measure the reaction rate of acetyl coenzyme A and oxaloacetic acid. Activity of HAD was measured using methods described previously (32), and rates were determined by calculating the rate of disappearance of NADH after the addition of acetoacetyl coenzyme A.

Statistical analyses. Repeated-measures analysis of variance was used to compare the data. Post-hoc analyses were performed using contrast-contrast analysis. Statistical significance was set at $p \leq 0.05$, and all data are expressed as mean ± SEM. Due to limitations in tissue size, all measurements could not be obtained for all individuals; the n for each variable is indicated.

Results

Subject characteristics. Anthropometric data are presented in Table 2.1 for the 12 lean and 10 obese men. Body mass, BMI, fasting insulin, and HOMA-IR were significantly higher in obese subjects ($p<0.01$). Blood lipids, glucose, and insulin did not change as a result of the high-fat diet or exercise training, and all subjects remained weight-stable throughout the duration of the study. The diet composition was similar between lean and obese subjects (72% fat, 15% carbohydrate, 13% protein), and provided a significant increase in dietary fat over their normal consumption recorded in a 3-day food log before commencement of the study which contained 35% fat, 48% carbohydrate, 17% protein.

Fatty acid oxidation in skeletal muscle. Fatty acid oxidation in response to the high-fat diet and exercise are presented in Fig. 2.2. The lean subjects increased complete palmitate oxidation ($^{14}CO_2$ production) by 27% in response to the 3 day HFD ($p=0.02$) indicating metabolic flexibility in response to an increase in ingested lipid. However, there was essentially no alteration in complete FAO in the obese group (1% increase vs. pre-diet values (Fig. 2.2), indicating a lack of metabolic flexibility. Exercise training ($p=0.02$) and 10d exercise plus 3d HFD ($p=0.002$) increased FAO above pre-exercise levels in both groups; however, in relation to metabolic flexibility, there was no significant increase in FAO with the addition of the high fat diet after the 7 days of exercise (Fig. 2.2). Total FAO (overall average 1450.8 nmol/mg protein/min) and ASM (overall average 1157.3 nmol/mg protein/min) did not change with the high-fat diet or exercise training, and were not different between lean and obese (data not shown).

Skeletal muscle enzyme activities/protein content. Enzyme activities for CS and HAD are presented in Fig. 2.3A and 2.3B, respectively. In the sedentary state, CS activity exhibited a pattern similar to complete FAO (Fig 2.3A), with a tendency for the lean subjects to increase (12.3±7.3%, p=0.17) in response to the high fat diet and obese to have an attenuated response (2.3±8.9% decrease from pre-diet values). Seven days of exercise training increased CS activity in both groups over the sedentary condition (p=0.02) with no further change at 10 days of exercise plus the 3-day high fat diet (p=0.03 compared to sedentary pre-diet). The trends for HAD responses to a high-fat diet and exercise training were similar to CS, with a high-fat diet+exercise increase that approached statistical significance (p=0.07 compared to sedentary pre-diet; Fig. 2.3B). Protein content of Complex II, III, IV, and ATP synthase subunits did not change with the high-fat diet or exercise training, and were not different between lean and obese individuals (Fig. 2.4A-D).

Discussion

The metabolic inflexibility evident with obesity was first reported by Kelley and colleagues, who observed an inability to increase glucose oxidation in response to euglycemic/hyperinsulinemic conditions in obese individuals (17). In relation to increased lipid presence with a high fat diet, both whole-body fat oxidation (1) and the transcription of genes regulating fat oxidation in skeletal muscle (3) did not increase in obese subjects compared to their lean counterparts. The ability to adjust FAO in response to lipid intake is a critical indicator of metabolic health, as inflexibility may lead to a positive fat balance (34) and excessive weight gain (26). For example, Zurlo and colleagues found that a dampening in the capacity to utilize lipid, as determined by whole-body RER, was associated with future weight gain in Pima Indians (39). In relation to intervention, lipid oxidation capacity in skeletal muscle can increase with relatively short-term (10d) endurance-oriented exercise training in both lean and obese individuals (2); however, it is not evident if exercise training can also rescue the metabolic inflexibility evident with obesity. The main findings of the present study were that: 1) obese, but relatively young individuals are metabolically inflexible in terms of increasing FAO capacity in skeletal muscle in response to a high fat diet and 2) exercise training increases the capacity for FAO in skeletal muscle in both lean and obese individuals, but does not dramatically potentiate metabolic flexibility.

In the current study, lean, but not obese, individuals increased skeletal muscle lipid oxidation capacity in response to the HFD (Fig. 2.2). This corresponds with other studies of lean and obese individuals at the whole-body level (1, 34). The handling of fuel substrates (glucose, fatty acids, and amino acids) in the cells and tissues of healthy, lean individuals is regulated by fuel sensors, which activate or inhibit metabolic pathways depending on the availability and

perceived needs of the cell. Increasing lipid availability in a lean, healthy individual, such as with several days of a HFD, activates pathways involved in increasing lipid oxidation to accommodate for this fatty acid overload and perhaps restore overall fat balance. Obese individuals have a diminished ability to increase lipid oxidation genes in response to a HFD (3), which suggests activation of these pathways is attenuated as well, thus contributing to positive lipid balance in skeletal muscle and other tissues (13).

A novel feature of our study was the inclusion of short-term aerobic exercise training as a means for improving skeletal muscle lipid oxidation capacity and perhaps metabolic flexibility. As predicted, we observed that 7d of exercise training increased skeletal muscle FAO to the same extent in both the lean and obese subjects (Fig. 2.2). Similar findings were found in lean, obese, and post-gastric bypass subjects (2), and in lean and obese Caucasian and African-American women (9) after 10d of exercise, suggesting that skeletal muscle lipid oxidation responds rapidly regardless of body habitus. However, no studies have looked at the effect of exercise training on metabolic flexibility, which would be the ability to potentiate FAO with the addition of a HFD. We saw that neither lean nor obese individuals increased their ability to elevate FAO in response to the HFD with exercise training (Fig. 2). However, exercise training increased FAO equivalent to or beyond the increment seen in response to the HFD alone (Fig. 2.2). These findings suggest that a high absolute capacity for FAO can render the muscle effective in terms of adjusting to increased dietary lipid.

Citrate synthase and HAD enzyme activities, along with electron transport chain protein content, were unchanged in response to the high-fat diet (Figs. 2.3A-B; 2.4A-D). Other studies have shown a HFD-induced increase in whole-body and skeletal muscle lipid oxidation in the absence of changes in indices of mitochondrial volume (14, 36), suggesting that different

mechanisms can be responsible for the high-fat diet-induced increases in lipid oxidation. Pyruvate dehydrogenase kinase 4 (PDK4), an enzyme that inhibits activity of the pyruvate dehydrogenase complex (PDH) and is crucial in the partitioning of carbohydrate and lipid substrates, appears to respond rapidly to increases in lipid (8). A high-fat (70% total calories) diet significantly increased PDK4 protein content and overall PDK activity in lean individuals after only one day (23), and PDK4 mRNA was increased in lean, but not obese individuals after a 5-day 60% fat diet (3). Increases in PDK4 protein are associated with reduced PDH activity (8) and increased lipid oxidation (6). Although limitations in tissue size prevented us from investigating PDK4 content and/or activity, differences in PDK4 content between lean and obese in response to exercise combined with a high-fat diet may help explain lipid oxidation responses to the intervention.

The activities of CS and HAD in response to exercise training mirrored the pattern of FAO changes we observed, with CS activity increasing after 7d of exercise and 10d exercise combined with the HFD and HAD tending to increase after combined diet and exercise (Fig. 2.3A-B). Aerobic exercise training has been shown to be a potent mechanism for improving maximal activity and content of mitochondrial proteins (5, 31, 33) in as little as 7-14d of training in lean individuals. Similar to our FAO data, we did not see an increase in enzyme activities with the addition of a HFD during exercise training, compared to exercise training alone (Fig. 2.3A-B). Although previous research in rats have shown an additive effect of exercise training and a HFD on CS and HAD activities in rodents (7, 29), these enzymes did not change with several weeks of a HFD in endurance-trained humans (14), even though they observed increases in whole-body lipid oxidation. This suggests perhaps that increased fat consumption exerts different effects on the mitochondria in humans compared to rodents. Nevertheless, as CS is

presumed to provide a reflection of overall mitochondrial content (37) the CS activity increases after training in our study may be an important factor contributing to increasing skeletal muscle FAO in obese individuals.

Protein content of electron transport chain enzymes were unchanged with a high-fat diet and exercise training, and were not different between lean and obese individuals (Fig. 2.4A-D). Exercise-induced adaptations in the mitochondria are thought to involve increases in the mRNA expression of transcription factors, nuclear receptors, and mitochondrial proteins 4-24h following a single exercise session (20, 25), although mitochondrial protein content does not change within this time frame (20). It is hypothesized that changes in protein content of transcription factors, such as peroxisome proliferator-activated receptor γ co-activator (PGC-1α), nuclear receptors such as the PPARs, and mitochondrial proteins, require an accumulation of these post-exercise mRNA bursts (12, 24, 38). Furthermore, the temporal pattern of expression may vary among the enzymes, as Perry and colleagues showed CS and HAD enzyme activities increased after 6d training, while COX IV protein did not show changes until 10d training (22). Since the Perry et al. study showed a significant 20% increase in COX IV protein after 10d exercise training, we expected to see increases in the protein content of COX IV and other subunits of electron transport chain proteins. However, the exercise training protocol of our study was relatively moderate (1h at 70% VO_2peak) compared to the one employed by Perry et al, who used a relatively high volume of interval training (10x4 min intervals at ~90% VO_2peak). Therefore, the higher intensity of the former protocol may have elicited a more robust response in the mitochondria compared to our study. More research should investigate the temporal response of mitochondrial proteins to exercise, as well as the potential influence of intensity on mitochondrial protein increases.

Extensive research has supported the ability of exercise training to improve lipid oxidation and mitochondrial capacity in the skeletal muscle of lean sedentary individuals (30, 33, 35). However, the ability of obese individuals to increase skeletal muscle mitochondrial capacity, and thus lipid oxidation, is controversial. One study showed that obese, insulin-resistant individuals failed to increase mRNA expression of PGC-1α and its downstream gene targets to the same extent as their lean counterparts after a single bout of aerobic exercise (10). However this study did not directly assess skeletal muscle lipid oxidation or whether this attenuated response continued after several days of training. Another study shows that a 16-20wk aerobic exercise training program increased mtDNA content in lean, but not obese individuals (27). Nevertheless, other studies support the hypothesis that obese individuals can increase skeletal muscle lipid oxidation in response to training: Bruce and colleagues showed a 120% increase in skeletal muscle lipid oxidation after eight wk of aerobic exercise training in obese (average BMI 36 kg/m^2) individuals (4). Furthermore, our previous research shows that the increase in skeletal muscle lipid oxidation in response to 10d training is similar between lean and obese individuals (2). The present findings support the concept that endurance-oriented exercise training can increase the capacity for FAO in the skeletal muscle of obese individuals and that this adaptation occurs to the same relative extent as in their lean counterparts.

In conclusion, 3d of a 70% eucaloric fat diet increased lipid oxidation in the skeletal muscle of lean individuals but did not alter FAO in obese individuals, indicating metabolic inflexibility in response to an increase in ingested lipid. Short-term aerobic exercise training increased lipid oxidation and CS activity similarly in both lean and obese individuals, although HFD-induced metabolic flexibility did not appear to improve in either group. Together, these findings suggest that the increase in FAO in skeletal muscle with endurance-oriented exercise

training may enable obese individuals to respond similarly to their lean counterparts when confronted with an increase in dietary lipid load.

Acknowledgements

The authors would like to thank Angela Clark, R.N. and Rita Bowden, R.N. for assisting with specimen collection and the undergraduate student assistants at for assisting with exercise training and testing.

Grants

Funding for this work was provided by a grant from the National Institutes of Health (DK 056112, JAH).

Disclosures

The authors have nothing to declare.

References

1. **Astrup A, Buemann B, Christensen NJ, and Toubro S**. Failure to increase lipid oxidation in response to increasing dietary fat content in formerly obese women. *Am J Physiol* 266: E592-599, 1994.
2. **Berggren JR, Boyle KE, Chapman WH, and Houmard JA**. Skeletal muscle lipid oxidation and obesity: influence of weight loss and exercise. *Am J Physiol Endocrinol Metab* 294: E726-732, 2008.
3. **Boyle KE, Canham JP, Consitt LA, Zheng D, Koves TR, Gavin TP, Holbert D, Neufer PD, Ilkayeva O, Muoio DM, and Houmard JA**. A high-fat diet elicits differential responses in genes coordinating oxidative metabolism in skeletal muscle of lean and obese individuals. *The Journal of clinical endocrinology and metabolism* 96: 775-781, 2011.
4. **Bruce CR, Thrush AB, Mertz VA, Bezaire V, Chabowski A, Heigenhauser GJ, and Dyck DJ**. 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-E107, 2006.
5. **Burgomaster KA, Hughes SC, Heigenhauser GJ, Bradwell SN, and Gibala MJ**. Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans. *J Appl Physiol* 98: 1985-1990, 2005.
6. **Cameron-Smith D, Burke LM, Angus DJ, Tunstall RJ, Cox GR, Bonen A, Hawley JA, and Hargreaves M**. A short-term, high-fat diet up-regulates lipid metabolism and gene expression in human skeletal muscle. *Am J Clin Nutr* 77: 313-318, 2003.
7. **Cheng B, Karamizrak O, Noakes TD, Dennis SC, and Lambert EV**. Time course of the effects of a high-fat diet and voluntary exercise on muscle enzyme activity in Long-Evans rats. *Physiology & behavior* 61: 701-705, 1997.
8. **Chokkalingam K, Jewell K, Norton L, Littlewood J, van Loon LJ, Mansell P, Macdonald IA, and Tsintzas K**. High-fat/low-carbohydrate diet reduces insulin-stimulated carbohydrate oxidation but stimulates nonoxidative glucose disposal in humans: An important role for skeletal muscle pyruvate dehydrogenase kinase 4. *The Journal of clinical endocrinology and metabolism* 92: 284-292, 2007.
9. **Cortright RN, Sandhoff KM, Basilio JL, Berggren JR, Hickner RC, Hulver MW, Dohm GL, and Houmard JA**. Skeletal muscle fat oxidation is increased in African-American and white women after 10 days of endurance exercise training. *Obesity* 14: 1201-1210, 2006.
10. **De Filippis E, Alvarez G, Berria R, Cusi K, Everman S, Meyer C, and Mandarino LJ**. Insulin-resistant muscle is exercise resistant: evidence for reduced response of nuclear-encoded mitochondrial genes to exercise. *Am J Physiol Endocrinol Metab* 294: E607-614, 2008.
11. **Flegal KM, Graubard BI, Williamson DF, and Gail MH**. Excess deaths associated with underweight, overweight, and obesity. *Jama* 293: 1861-1867, 2005.
12. **Fluck M, and Hoppeler H**. Molecular basis of skeletal muscle plasticity--from gene to form and function. *Reviews of physiology, biochemistry and pharmacology* 146: 159-216, 2003.
13. **Galgani JE, Moro C, and Ravussin E**. Metabolic flexibility and insulin resistance. *Am J Physiol Endocrinol Metab* 295: E1009-1017, 2008.
14. **Goedecke JH, Christie C, Wilson G, Dennis SC, Noakes TD, Hopkins WG, and Lambert EV**. Metabolic adaptations to a high-fat diet in endurance cyclists. *Metabolism: clinical and experimental* 48: 1509-1517, 1999.

15. **Harris JA, and Benedict FG**. A Biometric Study of Human Basal Metabolism. *Proc Natl Acad Sci U S A* 4: 370-373, 1918.
16. **Jong-Yeon K, Hickner RC, Dohm GL, and Houmard JA**. Long- and medium-chain fatty acid oxidation is increased in exercise-trained human skeletal muscle. *Metabolism: clinical and experimental* 51: 460-464, 2002.
17. **Kelley DE, Goodpaster B, Wing RR, and Simoneau JA**. Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity, and weight loss. *Am J Physiol* 277: E1130-1141, 1999.
18. **Kelley DE, and Mandarino LJ**. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes* 49: 677-683, 2000.
19. **Kim JY, Hickner RC, Cortright RL, Dohm GL, and Houmard JA**. Lipid oxidation is reduced in obese human skeletal muscle. *Am J Physiol Endocrinol Metab* 279: E1039-1044, 2000.
20. **Leick L, Plomgaard P, Gronlokke L, Al-Abaiji F, Wojtaszewski JF, and Pilegaard H**. Endurance exercise induces mRNA expression of oxidative enzymes in human skeletal muscle late in recovery. *Scandinavian journal of medicine & science in sports* 20: 593-599, 2010.
21. **Moro C, Galgani JE, Luu L, Pasarica M, Mairal A, Bajpeyi S, Schmitz G, Langin D, Liebisch G, and Smith SR**. Influence of gender, obesity, and muscle lipase activity on intramyocellular lipids in sedentary individuals. *The Journal of clinical endocrinology and metabolism* 94: 3440-3447, 2009.
22. **Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, and Spriet LL**. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J Physiol* 588: 4795-4810, 2010.
23. **Peters SJ, Harris RA, Wu P, Pehleman TL, Heigenhauser GJ, and Spriet LL**. Human skeletal muscle PDH kinase activity and isoform expression during a 3-day high-fat/low-carbohydrate diet. *Am J Physiol Endocrinol Metab* 281: E1151-1158, 2001.
24. **Pilegaard H, Ordway GA, Saltin B, and Neufer PD**. Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. *Am J Physiol Endocrinol Metab* 279: E806-814, 2000.
25. **Pilegaard H, Saltin B, and Neufer PD**. Exercise induces transient transcriptional activation of the PGC-1alpha gene in human skeletal muscle. *J Physiol* 546: 851-858, 2003.
26. **Ravussin E**. Metabolic differences and the development of obesity. *Metabolism: clinical and experimental* 44: 12-14, 1995.
27. **Ritov VB, Menshikova EV, Azuma K, Wood R, Toledo FG, Goodpaster BH, Ruderman NB, and Kelley DE**. Deficiency of electron transport chain in human skeletal muscle mitochondria in type 2 diabetes mellitus and obesity. *Am J Physiol Endocrinol Metab* 298: E49-58, 2010.
28. **Russell AP, Gastaldi G, Bobbioni-Harsch E, Arboit P, Gobelet C, Deriaz O, Golay A, Witztum JL, and Giacobino JP**. Lipid peroxidation in skeletal muscle of obese as compared to endurance-trained humans: a case of good vs. bad lipids? *FEBS letters* 551: 104-106, 2003.
29. **Simi B, Sempore B, Mayet MH, and Favier RJ**. Additive effects of training and high-fat diet on energy metabolism during exercise. *J Appl Physiol* 71: 197-203, 1991.
30. **Smith SR, de Jonge L, Zachwieja JJ, Roy H, Nguyen T, Rood J, Windhauser M, Volaufova J, and Bray GA**. Concurrent physical activity increases fat oxidation during the shift to a high-fat diet. *Am J Clin Nutr* 72: 131-138, 2000.

31. **Spina RJ, Chi MM, Hopkins MG, Nemeth PM, Lowry OH, and Holloszy JO**. Mitochondrial enzymes increase in muscle in response to 7-10 days of cycle exercise. *J Appl Physiol* 80: 2250-2254, 1996.
32. **Srere PA**. Citrate Synthase. In: *Methods in Enzymology*, edited by J L. New York: Academic Press, 1969.
33. **Talanian JL, Galloway SD, Heigenhauser GJ, Bonen A, and Spriet LL**. Two weeks of high-intensity aerobic interval training increases the capacity for fat oxidation during exercise in women. *J Appl Physiol* 102: 1439-1447, 2007.
34. **Thomas CD, Peters JC, Reed GW, Abumrad NN, Sun M, and Hill JO**. Nutrient balance and energy expenditure during ad libitum feeding of high-fat and high-carbohydrate diets in humans. *Am J Clin Nutr* 55: 934-942, 1992.
35. **Tunstall RJ, Mehan KA, Wadley GD, Collier GR, Bonen A, Hargreaves M, and Cameron-Smith D**. Exercise training increases lipid metabolism gene expression in human skeletal muscle. *Am J Physiol Endocrinol Metab* 283: E66-72, 2002.
36. **Vogt M, Puntschart A, Howald H, Mueller B, Mannhart C, Gfeller-Tuescher L, Mullis P, and Hoppeler H**. Effects of dietary fat on muscle substrates, metabolism, and performance in athletes. *Medicine and science in sports and exercise* 35: 952-960, 2003.
37. **Wang H, Hiatt WR, Barstow TJ, and Brass EP**. Relationships between muscle mitochondrial DNA content, mitochondrial enzyme activity and oxidative capacity in man: alterations with disease. *European journal of applied physiology and occupational physiology* 80: 22-27, 1999.
38. **Williams RSN, P. D.** Regulation of gene expression in skeletal muscle by contractile activity. In: *The Handbook of Physiology*, edited by JT RLS. New York: Oxford University Press, 1996, p. 1124-1150.
39. **Zurlo F, Lillioja S, Esposito-Del Puente A, Nyomba BL, Raz I, Saad MF, Swinburn BA, Knowler WC, Bogardus C, and Ravussin E**. Low ratio of fat to carbohydrate oxidation as predictor of weight gain: study of 24-h RQ. *Am J Physiol* 259: E650-657, 1990.

Tables and Figures

Table 2.1. Subject characteristics**.**

	Lean (n=12)	**Obese (n=10)**
Age (y)	21.8±1.1	22.4±0.8
Height (cm)	178.9±2.0	179.1±2.2
Mass (kg)	72.2±2.4	108.4±3.3*
BMI (kg/m^2)	22.6±0.7	33.7±0.7*
Body Fat (%) (DEXA)	17.7±1.8	37.5±1.8*
Fasting glucose (mmol/L)	4.7±0.1	4.7±0.1
Fasting insulin (uU/L)	6.4±1.2	11.3±1.2*
HOMA-IR	1.3±0.2	2.4±0.3*
Plasma cholesterol (mg/dl)	153±9	178±12
Plasma triglycerides (mg/dl)	86±9	113±12*
VO_2 peak (ml/kg/min)	36.7±1.2	27.2±1.2*
VO_2 peak (l/min)	2.6±0.2	2.9±0.2

Results are expressed as mean±SEM

*Significantly different ($P \leq 0.05$) from lean.

Figure 2.1. Study design. All subjects were screened and tested for maximal aerobic capacity before commencement of the study. Within 4 weeks of screening, subjects underwent a fasting muscle biopsy of the vastus lateralis, consumed an isocaloric 70% high fat diet (HFD) for 3d, and then had another muscle biopsy the morning after the final day of the HFD. After a 2-3 week washout period, subjects began exercise training for 10 consecutive days, 1h/d, @ 70% VO_2peak. On the morning of day 8, subjects had a muscle biopsy and began consuming the HFD during days 8-10 of exercise training. The day after finishing the exercise training and second HFD, subjects underwent their final muscle biopsy.

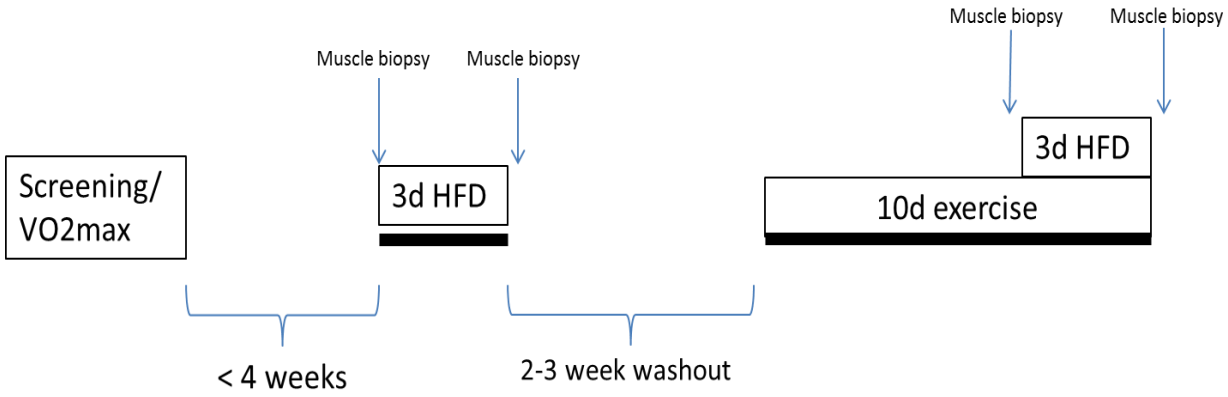


Figure 2.2. Complete palmitate oxidation ($^{14}CO_2$ production from palmitate) in skeletal muscle biopsies before (pre-diet) and following a 3-day high-fat diet in the sedentary condition (3d HFD, sedentary) for lean (n=9) and obese (n=8) males. After a 2 to 3-week washout period (indicated by hashed lines), subjects performed 10 consecutive days of aerobic exercise training and $^{14}CO_2$ production from palmitate was measured after 7 days of exercise and after a 3-day high-fat diet+ 10 days exercise. Results are expressed as mean ± SEM. *Significantly different from obese after the 3 day high fat diet (p=0.02); †Significantly increased compared to the pre-diet condition for lean (p=0.02); ‡Significant treatment effect for 7 days exercise compared to the pre-diet condition (p=0.02); §Significant treatment effect for the 10 day exercise + high fat diet compared to the pre-diet condition (p<0.01).

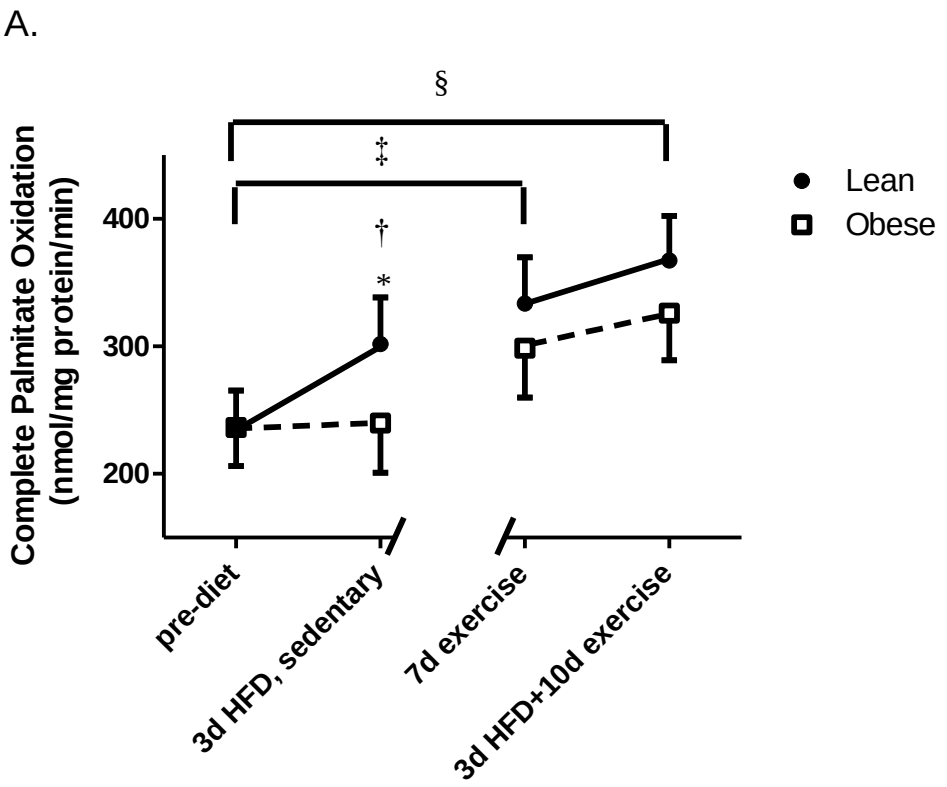
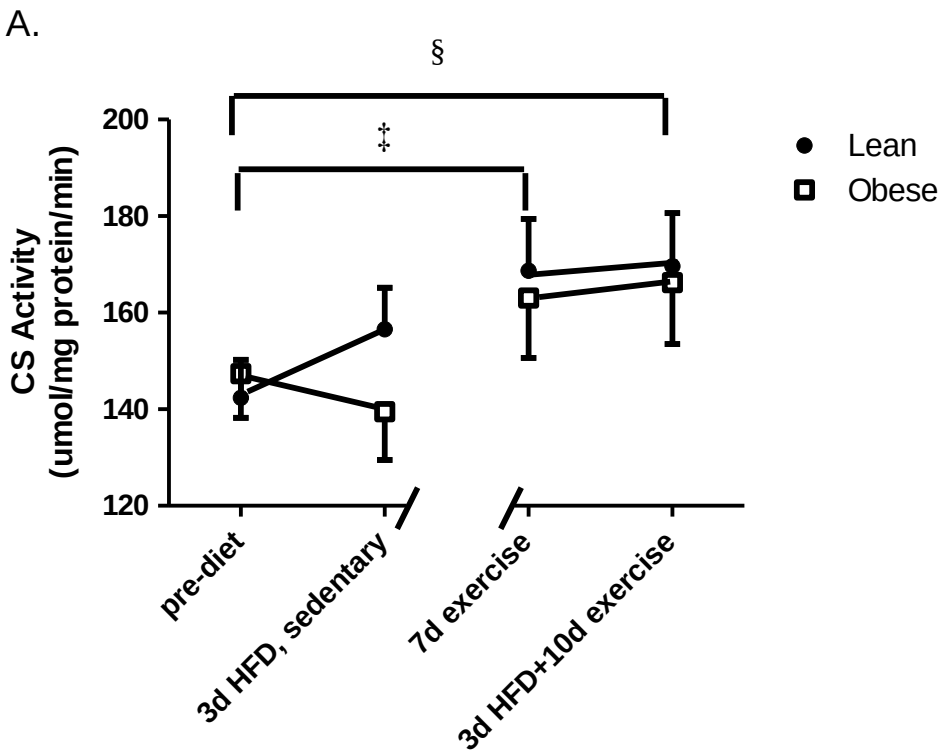


Figure 2.3. Citrate synthase (Panel A) and HAD (Panel B) pre-diet and after 3 days of a high-fat diet (HFD). After a 2 to 3-week washout period (indicated by hashed lines), subjects exercised for 10 consecutive days and enzyme activities were measured after 7 days exercise training and concurrent 10d exercise training and 3d HFD. CS activity was determined from 12 lean and 9 obese subjects, while HAD was determined from 10 lean and 8 obese subjects. Results are expressed as mean ± SEM. ‡Significant treatment effect for 7 days exercise compared to the pre-diet condition (p=0.02); §Significant treatment effect for the 10 day exercise + high fat diet compared to the pre-diet condition (p=0.03).



B.

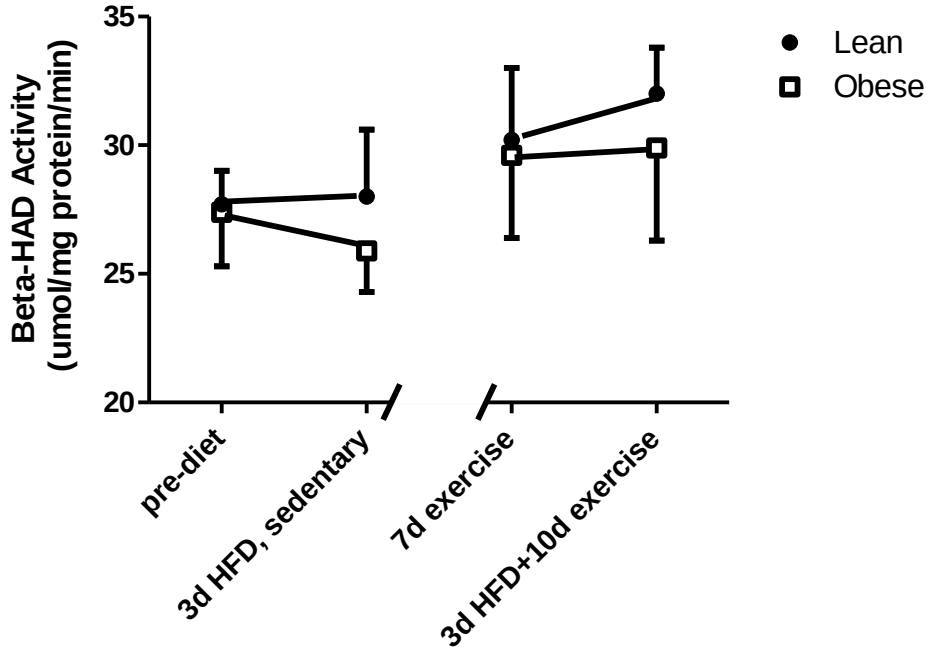
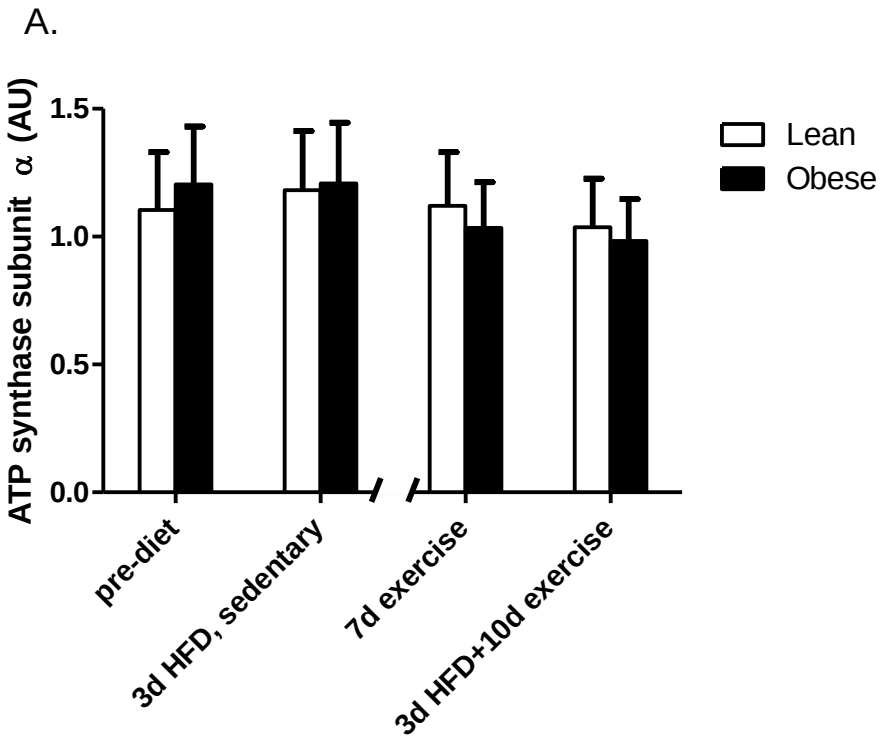
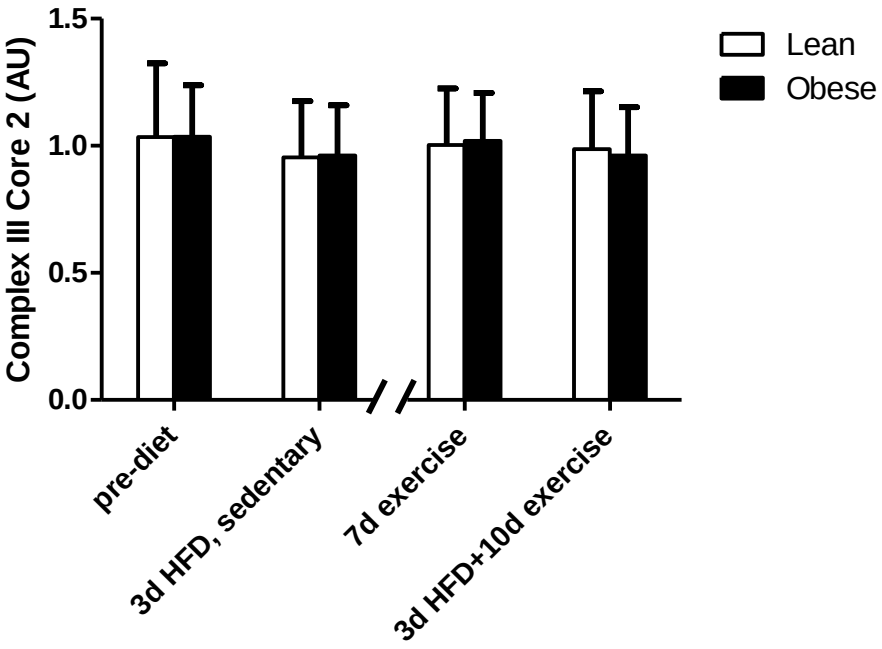

Beta-HAD Activity
(umol/mg protein/min)
35
30
25
20
pre-diet
3d HFD, sedentary
7d exercise
3d HFD+10d exercise
Lean
Obese

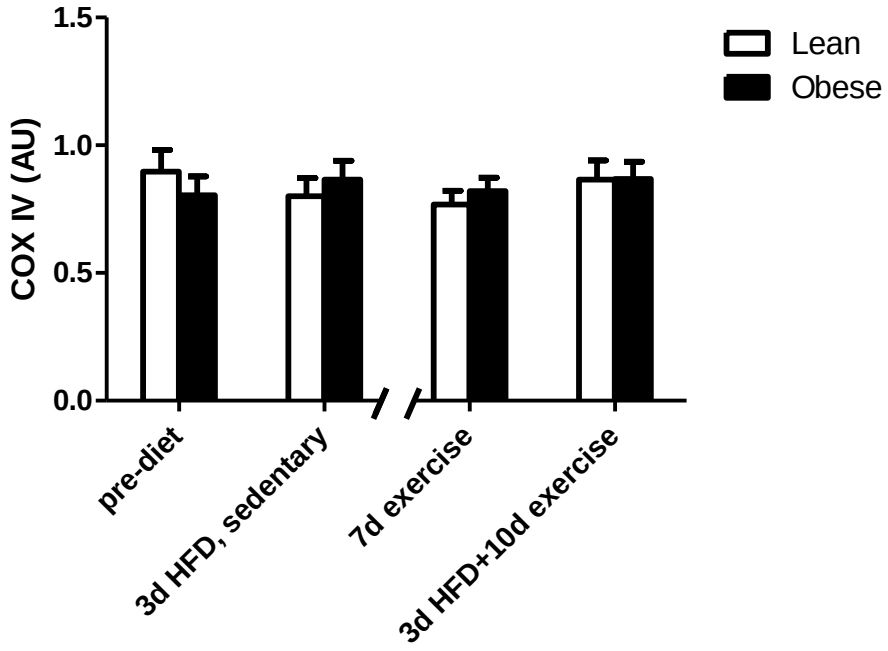
Figure 2.4. Protein content of mitochondrial electron transport chain enzyme subunits before and after a 3d HFD in the sedentary condition (pre-exercise), and before and after a 3d HFD consumed during days 8-10 of a 10d exercise training program. Panel A: ATP synthase subunit alpha; Panel B: Complex III Core 2; Panel C: COX IV; Panel D: Complex II 30kDa. Results are expressed as mean ± SEM.



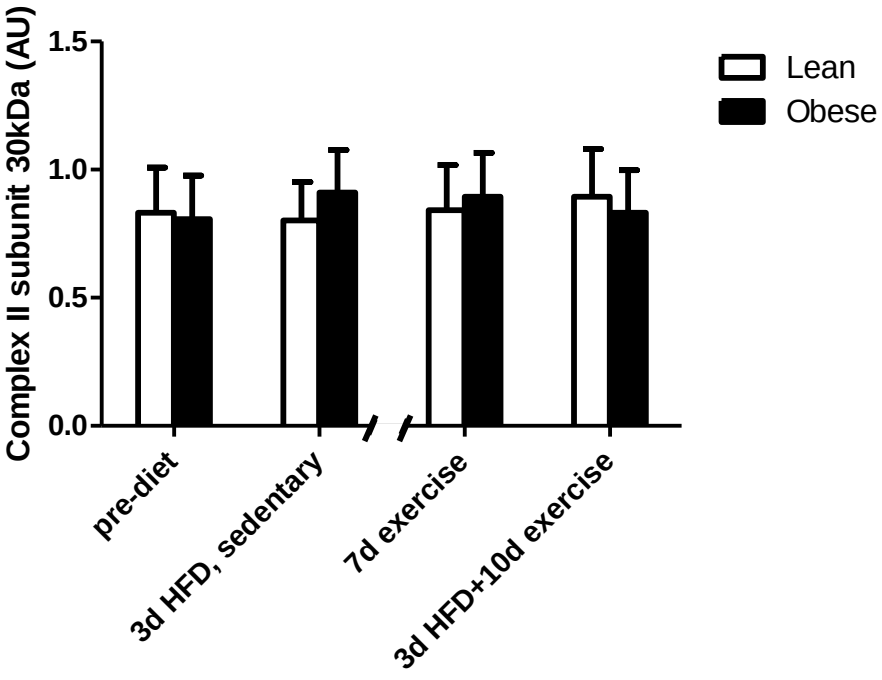
B.


Complex III Core 2 (AU)
1.5
1.0
0.5
0.0
pre-diet
3d HFD, sedentary
7d exercise
3d HFD+10d exercise
Lean
Obese

C.


1.5
1.0
0.5
0.0
COX IV (AU)
Lean
Obese
pre-diet
3d HFD, sedentary
7d exercise
3d HFD+10d exercise

D.


Complex II subunit 30kDa (AU)
1.5
1.0
0.5
0.0
Lean
Obese
pre-diet
3d HFD, sedentary
7d exercise
3d HFD+10d exercise

CHAPTER 3: EFFECTS OF INCREASED LIPID AND CELL ENERGY EXPENDITURE ON MITOCHONDRIAL CONTENT IN CULTURED MYOTUBES FROM LEAN AND OBESE HUMANS.

Abstract

Human skeletal muscle cells raised in culture (HSkMC) as well as skeletal muscle *in vivo* from obese individuals are characterized by an inability to increase mitochondrial content in response to increased lipid. Exercise training increases lipid oxidation in skeletal muscle which in turn enables lean and obese individual to respond equivalently to a high fat diet. The purpose of the present study was to determine if increasing substrate turnover via pharmacological uncoupling could augment mitochondrial content and in turn normalize the response to lipid (metabolic flexibility) in obese skeletal muscle in a manner similar to exercise. HSkMC from 10 lean (BMI 22.1±0.8 kg/m^2) and 10 obese (BMI 35.5±1.9 kg/m^2) were examined under the following conditions: 1) Control, 2) 24h lipid incubation (FAT), 3) 72h incubation with the chemical uncoupler FCCP (FCCP), and 4) 48h of FCCP +24h combined FCCP and lipid (FCCP+FAT). Protein content of OXPHOS enzymes, citrate synthase activity, and respiration rates were measured. FAT incubation increased Complex I, Complex III, and COX IV protein content in lean, but not obese HSkMC. FCCP and FCCP+FAT increased Complex I irrespective of obesity status, while COX IV and Complex III ($p=0.08$) increased in only the lean subjects. Basal and maximal O_2 consumption increased with FCCP+FAT. These findings indicate that the skeletal muscle of obese individuals exhibits a decrement in the ability to increase the expression of key OXPHOS proteins with lipid exposure which is not completely rescued with accelerated cell respiration. Keywords: mitochondria, skeletal muscle, uncoupling, fatty acid

Introduction

Obesity is increasing in its prevalence and is associated with metabolic dysfunction and disease (8). An important indicator of metabolic health is metabolic flexibility, which is the ability to appropriately adjust substrate utilization based on availability (10). With obesity there are impairments in metabolic flexibility in response to increased lipid presence, as indicated by a dampened ability to increase whole-body lipid oxidation (1) as well as the transcription of genes involved in lipid metabolism in skeletal muscle (5) with the imposition of a high-fat diet. Human skeletal muscle cells raised in culture (HSkMC) from obese donors also display an inability to increase lipid-fueled cell respiration and the expression of proteins linked with fatty acid oxidation in response to prolonged (24h) lipid incubation (6) indicating that this impairment in metabolic flexibility is a resilient and persistent trait.

In terms of intervention, endurance-oriented exercise training increases lipid oxidation in skeletal muscle (2) which permits obese individuals to respond similarly to a high fat diet as lean subjects (Battaglia et al, in review). In muscle, exercise induces multiple acute alterations that promote mitochondrial biogenesis, including increased AMP:ATP ratio, oscillations in intracellular Ca^{2+}, increases in reactive oxygen and nitrogen species, and mechanical stress (12, 17). The increase in mitochondrial content with exercise results in a more oxidative phenotype, which may enhance the ability to appropriately regulate lipid oxidation when exposed to excess lipid substrate (12). In respect to discerning cellular mechanisms by which exercise rescues metabolic flexibility, uncoupling of the proton gradient into the mitochondrial matrix may accelerate substrate use and cell respiration in a manner that may be similar to aerobic exercise. In 10T ½ fibroblasts 72h incubation with the chemical uncoupler carbonyl cyanide p-(triflurometoxy) phenyl hydrazone (FCCP) increased the expression of genes involved in

peroxisome proliferator-activated receptor-coactivator-1α (PGC-1α) mediated mitochondrial biogenesis (18). However, FCCP has a narrow functional range outside of which leads to cell death (13), and the dose of FCCP used in this study was 25 to 50-fold higher than the dose used to elicit maximal cell respiration in human myotubes. Furthermore, since the study measured transcription of genes involved in mitochondrial biogenesis, it is unknown if FCCP treatment results in functional increases in mitochondrial protein content and cell respiration capacity, thus enabling the cell to respond to high lipid presence. The intent of the present study was to determine if accelerating mitochondrial respiration rate via pharmacological uncoupling is one of the critical factors that increases mitochondrial content and restores metabolic flexibility in the skeletal muscle of obese individuals with lipid overload, i.e. in a manner akin to endurance-oriented exercise. This was accomplished by 1) determining if HSkMC from obese donors exhibit an impaired response to lipid incubation in terms of increasing mitochondrial content and cell respiration, and 2) whether increasing substrate turnover within the cell could rescue any observed impairments in metabolic flexibility with obesity.

Methods

Study design. The intent of this study was to determine if pre-treatment with a chemical uncoupler (FCCP) improved metabolic flexibility to lipid overload in HSkMC from lean and obese donors. Satellite cells were derived from muscle biopsies and were subsequently differentiated into myotubes as described previously (3). Cells from each subject were divided into multiple wells, which were subdivided into the following four conditions (Fig. 3.1): Control, 1:1 100 µM oleate:palmitate incubation over the final 24h of differentiation (FAT), 400 nM FCCP (FCCP) over the final 72h of differentiation, and 48h of FCCP incubation+24h combined FCCP and lipid (FCCP+FAT). After the treatments, western blots of OXPHOS enzymes (Complex I subunit NDUFB8, Complex II subunit 30kDa, Complex III subunit Core 2, cytochrome c oxidase subunit IV, and ATP synthase subunit alpha) and citrate synthase enzyme activity were used as indices of mitochondrial content. Cell respiration was determined using a Seahorse Bioscience XF24 Extracellular Flux Analyzer.

Subjects. Skeletal muscle was obtained from the vastus lateralis in 10 lean (BMI 22.1 ± 0.8 kg/m^2) and 10 obese (BMI 35.5 ± 1.9 kg/m^2) males age 18 - 26y. Participants filled out a medical history questionnaire to confirm they were free from disease, did not smoke, and were not taking any medications known to influence carbohydrate or lipid metabolism. Subjects were weight-stable (± 2 kg over the past 3 months) and nonsmokers. The experimental procedure and associated risks were explained in detail in written and oral format and informed consent obtained. The study was approved by the East Carolina Policy and Review Committee on Human Research and was in accordance with the Declaration of Helsinki.

Cell Culture. Satellite cells were isolated from 50-100 mg of muscle tissue as previously described (3). Cells were thawed and sub-cultured in T-75 flasks and at 80-90% confluence, were lifted with 0.05% trypsin EDTA, and centrifuged for 10 min. The resuspended pellet was plated onto 6-well type I collagen-coated plates and 24-well Seahorse XF plates (Seahorse Bioscience, North Billerica, MA) at a density of $8x10^5$ and $1x10^5$ cells per well, respectively. At 70-80% confluence, differentiation was initiated by replacing growth media with differentiation media, which consisted of Dulbecco Modified Eagle's Medium (DMEM) supplemented with 2% horse serum, 0.5 mg/ml fatty acid-free bovine serum albumin (BSA), 0.5 mg/ml fetuin, and 50 ug/ml gentamycin/amphotericin B. Differentiation media was changed every 48h. On day 4, fresh differentiation media containing 0.04% molecular-grade ethanol was added to the control and FAT wells, and media containing 400nM FCCP dissolved in ethanol was added to the FCCP and FCCP+FAT wells. On day 6, fresh differentiation media containing 0.04% ethanol and 0.5% BSA (control), 0.04% ethanol and 100 µM 1:1 oleate:palmitate treatment (FAT), 400 nM FCCP and 0.5% BSA (FCCP), and 400 nM FCCP and 100µM oleate:palmitate (FCCP+FAT) was added to the respective wells. Cells were harvested on day 7 for experiments.

Dose response. High doses of FCCP can result in impaired ATP production and cell death (13). Furthermore, while 72h incubation with 25 µM FCCP in 10T ½ fibroblasts increased mRNA transcripts of genes involved in mitochondrial biogenesis (18), we found reduced whole-cell respiration rates in response to an acute injection of 2-4 µM FCCP in HSkMC (data not shown). Therefore, we measured lipid oxidation and mitochondrial protein content in response to 72h FCCP incubations with a variety of concentrations lower than 1 µM, with the intent of finding the optimal concentration for eliciting maximal increases in lipid oxidation and mitochondrial content. Lipid oxidation increased significantly with 200 nM ($p=0.003$, 1.7-fold increase over

basal) and 400 nM FCCP (p=0.03, 1.7-fold increase over basal) incubations compared to control cells, with no increase in lipid oxidation at a concentration of 600 nM FCCP (Fig. 3.3). Protein content for the majority of mitochondrial enzymes (Complex I subunit NDUFB8, Complex II subunit 30kDa, Complex III subunit Core 2, cytochrome c oxidase subunit IV, and ATP synthase subunit alpha) and citrate synthase activity increased optimally at the 400 nM concentration (Fig. 3.4A-F). Based on this information, 400 nM FCCP was used as the concentration for all subsequent studies.

Lipid oxidation. On day 7 of differentiation, myotubes were incubated for 3h at 37°C in sealed 24-well plates containing differentiation media, 12.5 mmol/l HEPES, 0.5% BSA, 1 mmol/l L-carnitine, 100 µmol/l sodium oleate (Sigma-Aldrich, St Louis, MO), and 1 µCi/ml [^{14}C] oleate (PerkinElmer, MA). Following incubation, the medium was assayed for $^{14}CO_2$ to measure complete fatty acid oxidation as previously described (14). Cells were washed twice with PBS, harvested in 200µl of 0.05% SDS lysis buffer, and stored at -80°C for determination of protein concentration.

Protein content. Cells were harvested in 100 µl ice-cold lysis buffer (50 mM HEPES [pH 7.4], 10 mM EDTA, 100 mM NaF, 12 mM Na pyrophosphate, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Samples were sonicated, rotated end-over-end at 4°C for 1h, and centrifuged at 20,000g for 20 min at 4°C. Protein concentrations were determined using the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL). Twenty micrograms of protein were separated by SDS-PAGE and electrotransferred to polyvinylidene flouride (PVDF) membranes (Millipore, Billerica, MA) and probed overnight for cytochrome c oxidase (COX) IV (1:1000; Cell Signaling, Beverly, MA) and with a cocktail

containing antibodies against the following proteins (1:1000): Complex I subunit NDUFB8, Complex II subunit 30kDa, Complex III subunit Core 2, Complex IV subunit II, and ATP synthase subunit alpha (Mitosciences, Eugene, OR; Fig. 3.2). Membranes were incubated for 1h at room temperature with the corresponding secondary antibody and the immunoreactive proteins were detected using enhanced chemiluminescence (ChemiDoc XRS+ Imaging System, BioRad Laboratories, Inc., Hercules, CA). Samples were normalized to a crude cell sample on each gel to normalize for blotting efficiency across gels.

Citrate synthase activity. Cells were harvested in 100 µl of a buffer containing 100 mM KH_2PO_4, 0.05% bovine serum albumin, and 0.08% Triton X-100. Samples were centrifuged at 20,000g for 20 min at 4°C. Protein concentrations were determined using a BCA assay kit, and all samples were diluted to 1.0 ug/ul using potassium phosphate buffer. Citrate synthase activity was determined using reagents provided in a kit (Sigma CS0720, St. Louis, MO), which used a colorimetric reaction to measure the reaction rate between acetyl coenzyme A and oxaloacetic acid.

Cellular respiration. On day 7 of differentiation, cell oxygen uptake rates (OCR) and extracellular acidification rates (ECAR) were measured in real-time using a Seahorse Bioscience XF24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA). Cells were washed with unbuffered DMEM, and preincubated for 1h at 37°C without CO_2 in 600 µL of unbuffered DMEM assay media (pH 7.4) supplemented with 1 mM sodium pyruvate and 200 µM 1:1 oleate:palmitate prior to the measurements. Following basal rates (OCR and ECAR in response to presence of substrate only), 2,4 dinitrophenol (DNP) (Sigma-Aldrich, St. Louis, MO)

was injected to a final concentration of 1 μM to measure maximal OCR rate under uncoupled conditions as an index of possible changes in total mitochondrial content.

Statistical analyses. Statistical analyses were performed using IBM SPSS Statistics 19 software (IBM Headquarters, Armonk, NY). Repeated-measures analysis of variance was used to compare the data. Post-hoc analyses were performed using contrast-contrast analysis. Statistical significance was set at $p < 0.05$, and all data are expressed as mean ± SEM.

Results

Subject characteristics. Subject characteristics are presented in Table 3.1. By design, obese individuals were heavier and had a significantly higher body mass index (BMI) than lean subjects ($p<0.001$). The obese group were also insulin-resistant compared to their lean counterparts, as indicated by HOMA-IR ($p=0.01$), and had significantly higher fasting insulin and total cholesterol levels ($p=0.01$ and 0.03, respectively). The obese group was also slightly older (22.4±0.8y) than the lean group (19.9±0.7y; $p=0.02$).

Mitochondrial Content

Lipid Treatment. Indices of mitochondrial content in response to 24h incubation with 100 µM 1:1 oleate:palmitate are presented in Fig. 3.5A. Indicative of metabolic flexibility, myotubes from lean individuals significantly increased the content of Complex I ($p=0.05$), Complex III ($p=0.01$), and COXIV subunits ($p=0.04$) in response to lipid incubation. In contrast, there were no changes in protein content in the obese individuals. Protein content of Complex II, Complex V, and citrate synthase activity did not change with lipid incubation in either of the subject groups.

FCCP Treatment. The 72h FCCP incubation significantly increased Complex I protein content ($p=0.03$) and Complex III protein content ($p=0.005$) (Fig. 3.5B) regardless of subject group (treatment effect). For Complex III, the increase in protein content tended to be greater ($p=0.08$) in cells from lean subjects (22% increase from control) than obese cells (6% increase from control). COXIV protein increased in the cells from lean ($p=0.02$) but not obese donors with FCCP treatment. Similar to the lipid incubation condition, there were no significant increases in

citrate synthase activity or protein content of the Complex II and Complex V enzyme subunits after the 72h FCCP incubation.

FCCP +Lipid Treatment. Similar to the FCCP only condition, the combined FCCP and lipid treatment significantly increased protein content of the Complex I ($p=0.02$) and Complex III subunits ($p=0.04$) (Fig. 3.5C). As with the FCCP treatment alone, the increase in Complex III was primarily driven by the lean subjects as the difference between the lean and obese subjects approached significance ($p=0.08$). Similar to the FCCP alone treatment, there was a differential response of COXIV with the lean subjects increasing and no change in the obese ($p=0.04$). Citrate synthase activity and the protein content from Complex II and V enzymes did not change with combined FCCP and lipid treatment.

Oxygen Consumption. Substrate-only and DNP-stimulated oxygen consumption rates are presented in Fig. 3.6A-B. Oxygen consumption rates with the substrate only were higher after the 72 hr combined FCCP and lipid incubation compared to all other conditions ($p<0.05$). With DNP-stimulation, lipid-treated cells had higher oxygen consumption rates than control cells ($p=0.01$). Combined lipid and FCCP treatment increased DNP-stimulated oxygen consumption over Control ($p=0.03$), lipid-only ($p=0.03$), and FCCP-only ($p<0.001$) conditions. Oxygen consumption responses did not differ between lean and obese for any of the conditions. Extracellular acidification rates did not change in response to any of the conditions and were not different between lean or obese (data not shown).

Discussion

The skeletal muscle of obese individuals does not appear to respond appropriately to an increase in lipid availability. For example, HSkMC from obese individuals do not alter lipid-fueled cell respiration following a 24h lipid incubation in contrast to the increase seen in cells from their lean counterparts (6). Similarly, exposure to a high-fat diet increased lipid oxidation and the expression of genes linked with mitochondrial biogenesis in the skeletal muscle of lean, but not obese subjects (5). In terms of intervention, both lean and obese individuals responded similarly to a high fat diet in terms of fatty acid oxidation in skeletal muscle after 10 consecutive days of endurance training (Battaglia et al, in review), and exercise training increased fatty acid oxidation and the mRNA content of genes linked with mitochondrial biogenesis regardless of obesity status (2). The intent of the present study was to determine whether accelerating mitochondrial respiration via chemical uncoupling in HSkMC could increase mitochondrial content and rescue the impaired ability to respond to lipid exposure in obese individuals in a manner resembling exercise training. The primary findings were that 1) lean, but not obese, individuals increased the content of proteins involved in oxidative phosphorylation (Complex I, Complex III, COXIV) in response to lipid incubation (FAT condition), consistent with impairments in metabolic flexibility with obesity and 2) chemical uncoupling (FCCP treatment) and chemical uncoupling + lipid incubation (FCCP + FAT) increased Complex I and Complex III content irrespective of body habitus, while COXIV only increased in myotubes from lean individuals. Therefore, under the experimental conditions utilized, elevating mitochondrial respiration via pharmacological uncoupling appears to rescue some, but not all aspects of the impairments in metabolic flexibility in skeletal muscle evident with obesity.

The activities of Complexes I and III have been shown to be rate-limiting in the oxidation of NADH (4) and Complex I, III, and COX enzymes may be associated in a "supramolecular assembly," whereas other enzymes are organized more randomly (reviewed in (11)). Thus, the selective increases in Complex I, III, and COXIV content after lipid incubation (FAT) in the lean subjects may allow for more efficient electron transfer throughout the mitochondrial respiratory chain, enabling the cell to adapt to increased lipid and ensuring metabolic flexibility (Fig. 3.5A). Furthermore, COXIV, a nuclear-encoded subunit of cytochrome c oxidase, is a frequently used index of mitochondrial content because of its role in regulating rates of respiration and ATP synthesis (9). However, while Complexes I, III, and COXIV content increased in response to increased lipid presence in myotubes from lean donors, none of these proteins increased in the obese individuals (Fig. 3.5A), which is supportive of our previous observations (6) indicating an impairment in the regulation of genes involved in oxidative metabolism in the skeletal muscle of obese individuals in response to lipid.

A novel component of the present study involved incubating myotubes with the chemical uncoupler FCCP in an attempt to mimic the increased substrate turnover of *in vivo* exercise and possibly rescue metabolic flexibility in obese tissue. An FCCP treatment effect was observed for Complexes I and III in HSkMC from both lean and obese donors, although the increase in Complex III was driven primarily by the lean subjects (Fig. 3.5B). Furthermore, only the lean individuals increased COXIV protein in response to FCCP incubation (Fig. 3.5B). These data are supportive of other findings indicating that the skeletal muscle of the obese can exhibit a dampened response to aerobic exercise in terms of triggering signals for mitochondrial biogenesis (7). However, exercise training can increase lipid oxidation (2) and mitochondrial density (21), indicating that the skeletal muscle of the obese are also fully capable of responding

to the intervention. Chemical uncoupling can activate AMPK and increase intracellular Ca^{2+} levels in fibroblasts (18) but cannot fully mimic all aspects of exercise, such as mechanical contraction and oscillations in intracellular Ca^{2+} flux. Under the experimental conditions utilized, the current findings suggest that convergence of these multiple factors, which occurs during muscle contraction, may be needed to elicit equivalent adaptive responses in the skeletal muscle of both lean and obese individuals.

Although previous research showed that incubation with 25 μM FCCP for 72h increased mRNA transcripts of PGC-1α, NRF-1, and OXPHOS proteins in 10T ½ fibroblasts (18), we observed that 400 nM FCCP for 72h was the optimal concentration for increasing lipid oxidation (Fig. 3.3) and mitochondrial proteins (Fig. 3.4A-F) in HSkMC. FCCP disrupts the proton motive force that drives ATP synthesis and while the cell is able to increase cell respiration in response to a low dose, too high a concentration will ultimately lead to cell death (13). The present study showed trends for lipid oxidation (Fig. 3.3) and mitochondrial protein (Fig. 3.4A-F) to decrease with incubation with 600nM FCCP for 72h. This suggests that the optimal physiological concentration of FCCP that depolarizes the mitochondrial membrane to trigger an increase in substrate utilization, while keeping the majority of cells viable, at least in primary human skeletal muscle cells is much lower than 25 μM.

Differential responses of certain electron transport chain proteins to lipid exposure remained evident with the combined FCCP+FAT treatment (Fig. 3.5C), indicating that uncoupling did not fully restore this aspect of metabolic flexibility in the muscle of obese individuals. However, combined FCCP+FAT incubation led to a significant increase in maximally stimulated respiration rate in intact myotubes from both lean and obese individuals

(Fig. 3.6B). Thus, maximal cellular respiration in the intact cell did not mirror the results evident in respiratory chain protein content, suggesting that other mechanisms, such as an improvement in mitochondrial function, are involved in controlling respiration rate.

Unlike the components of the respiratory system that were altered with lipid and/or chemical uncoupling in the lean subjects (Complex I, III, and IV) (Fig. 3.5A-C), Complex II, which accepts electrons via $FADH_2$, does not create a proton gradient as it transfers electrons through the electron transport chain (11). Pharmacological uncoupling dissipates the proton gradient, which may induce adaptations in proteins linked with this process rather than Complex II (Fig. 3.5B-C). Unlike Complex I, III, IV, and ATP synthase, which require coordination of nuclear and mitochondrial genomes and are regulated by multiple assembly factors, the four subunits of Complex II are only encoded by nuclear genes, and the roles for Complex II assembly factors are not clear. It is postulated that perhaps the cell preferentially dedicates proteins for synthesis of the other complexes rather than Complex II (19), and thus may explain the lack of Complex II response to FCCP and/or FAT that we observed (Fig. 3.5A-C). Furthermore, citrate synthase (CS) activity, which has been shown to respond to as little as 6-10 d of endurance-oriented exercise training in human skeletal muscle (16, 20), did not change with FAT and/or FCCP incubation (Fig. 3.5A-C). The lack of CS activity response to FAT incubation agrees with previous findings on CS activity in response to lipid incubation in human myotubes (6, 15), suggesting that high lipid load does not trigger an increase in CS activity *in vitro*. As FCCP targets the electron transport chain, it may specifically induce changes in the content and/or activity of enzymes involved as opposed to other energy producing pathways such as the TCA cycle.

In conclusion, the present findings indicate that the skeletal muscle of obese individuals exhibits a blunted response to lipid in terms of increasing components of the respiratory chain (Complex I & III and COXIV). These data support our previous findings (5, 6) of metabolic inflexibility in human skeletal muscle with obesity. Accelerating mitochondrial respiration via incubation with a chemical uncoupling agent (FCCP) did not fully restore the ability to increase the expression of the respiratory proteins in response to lipid incubation in obese individuals, indicating that other *in vitro* intervention strategies are likely needed to mimic the positive benefits of *in vivo* exercise training in obese individuals.

Acknowledgements

The authors would like to thank Angela Clark, R.N. and Rita Bowden, R.N. for assisting with specimen collection.

Grants

Funding for this work was provided by a grant from the National Institutes of Health (DK 056112, JAH).

Disclosures

The authors have nothing to declare.

References

1. **Astrup A, Buemann B, Christensen NJ, and Toubro S**. Failure to increase lipid oxidation in response to increasing dietary fat content in formerly obese women. *Am J Physiol* 266: E592-599, 1994.
2. **Berggren JR, Boyle KE, Chapman WH, and Houmard JA**. Skeletal muscle lipid oxidation and obesity: influence of weight loss and exercise. *Am J Physiol Endocrinol Metab* 294: E726-732, 2008.
3. **Berggren JR, Tanner CJ, and Houmard JA**. Primary cell cultures in the study of human muscle metabolism. *Exerc Sport Sci Rev* 35: 56-61, 2007.
4. **Bianchi C, Genova ML, Parenti Castelli G, and Lenaz G**. The mitochondrial respiratory chain is partially organized in a supercomplex assembly: kinetic evidence using flux control analysis. *J Biol Chem* 279: 36562-36569, 2004.
5. **Boyle KE, Canham JP, Consitt LA, Zheng D, Koves TR, Gavin TP, Holbert D, Neufer PD, Ilkayeva O, Muoio DM, and Houmard JA**. A high-fat diet elicits differential responses in genes coordinating oxidative metabolism in skeletal muscle of lean and obese individuals. *The Journal of clinical endocrinology and metabolism* 96: 775-781, 2011.
6. **Boyle KE, Zheng D, Anderson EJ, Neufer PD, and Houmard JA**. Mitochondrial lipid oxidation is impaired in cultured myotubes from obese humans. *International journal of obesity* 2011.
7. **De Filippis E, Alvarez G, Berria R, Cusi K, Everman S, Meyer C, and Mandarino LJ**. Insulin-resistant muscle is exercise resistant: evidence for reduced response of nuclear-encoded mitochondrial genes to exercise. *Am J Physiol Endocrinol Metab* 294: E607-614, 2008.
8. **Flegal KM, Graubard BI, Williamson DF, and Gail MH**. Excess deaths associated with underweight, overweight, and obesity. *Jama* 293: 1861-1867, 2005.
9. **Fontanesi F, Soto IC, Horn D, and Barrientos A**. Assembly of mitochondrial cytochrome c-oxidase, a complicated and highly regulated cellular process. *Am J Physiol Cell Physiol* 291: C1129-1147, 2006.
10. **Kelley DE, and Mandarino LJ**. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes* 49: 677-683, 2000.
11. **Lenaz G, and Genova ML**. Structure and organization of mitochondrial respiratory complexes: a new understanding of an old subject. *Antioxidants & redox signaling* 12: 961-1008, 2010.
12. **Lira VA, Benton CR, Yan Z, and Bonen A**. PGC-1alpha regulation by exercise training and its influences on muscle function and insulin sensitivity. *Am J Physiol Endocrinol Metab* 299: E145-161, 2010.
13. **Lou PH, Hansen BS, Olsen PH, Tullin S, Murphy MP, and Brand MD**. Mitochondrial uncouplers with an extraordinary dynamic range. *The Biochemical journal* 407: 129-140, 2007.
14. **Muoio DM, Way JM, Tanner CJ, Winegar DA, Kliewer SA, Houmard JA, Kraus WE, and Dohm GL**. Peroxisome proliferator-activated receptor-alpha regulates fatty acid utilization in primary human skeletal muscle cells. *Diabetes* 51: 901-909, 2002.
15. **Ortenblad N, Mogensen M, Petersen I, Hojlund K, Levin K, Sahlin K, Beck-Nielsen H, and Gaster M**. Reduced insulin-mediated citrate synthase activity in cultured skeletal muscle cells from patients with type 2 diabetes: evidence for an intrinsic oxidative enzyme defect. *Biochimica et biophysica acta* 1741: 206-214, 2005.

16. **Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, and Spriet LL**. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J Physiol* 588: 4795-4810, 2010.
17. **Rockl KS, Witczak CA, and Goodyear LJ**. Signaling mechanisms in skeletal muscle: acute responses and chronic adaptations to exercise. *IUBMB life* 60: 145-153, 2008.
18. **Rohas LM, St-Pierre J, Uldry M, Jager S, Handschin C, and Spiegelman BM**. A fundamental system of cellular energy homeostasis regulated by PGC-1alpha. *Proc Natl Acad Sci U S A* 104: 7933-7938, 2007.
19. **Rutter J, Winge DR, and Schiffman JD**. Succinate dehydrogenase - Assembly, regulation and role in human disease. *Mitochondrion* 10: 393-401, 2010.
20. **Spina RJ, Chi MM, Hopkins MG, Nemeth PM, Lowry OH, and Holloszy JO**. Mitochondrial enzymes increase in muscle in response to 7-10 days of cycle exercise. *J Appl Physiol* 80: 2250-2254, 1996.
21. **Toledo FG, Menshikova EV, Azuma K, Radikova Z, Kelley CA, Ritov VB, and Kelley DE**. Mitochondrial capacity in skeletal muscle is not stimulated by weight loss despite increases in insulin action and decreases in intramyocellular lipid content. *Diabetes* 57: 987-994, 2008.
22. **Zuurveld JG, Oosterhof A, Veerkamp JH, and van Moerkerk HT**. Oxidative metabolism of cultured human skeletal muscle cells in comparison with biopsy material. *Biochimica et biophysica acta* 844: 1-8, 1985.

Tables and Figures

Table 3.1 Subject Characteristics

	Lean (n=10)	**Obese (n=10)**
Age (y)	19.9±0.7	22.4±0.8*
Height (cm)	177.2±1.7	179.5±2.1
Mass (kg)	69.3±2.4	114.9±6.8*
BMI (kg/m^2)	22.1±0.8	35.5±1.9*
Fasting glucose (mmol/L)	4.6±0.1	4.8±0.1
Fasting insulin (uU/L)	6.8±1.0	12.6±1.9*
HOMA-IR	1.4±0.2	2.7±0.4*
Plasma cholesterol (mg/dl)	136±7	164±10*
Plasma triglycerides (mg/dl)	75±9	118±21

Values are expressed as mean ± SEM.

*Significantly different from lean ($p<0.05$).

Fig. 3.1 Study Design. Myotubes cultured from lean and obese donors were differentiated at 70-80% confluence and incubated under the following conditions: Differentiation media supplemented with 2% horse serum (Control); 24h 100 µM 1:1 oleate:palmitate (FAT); 72h 400 nM FCCP (FCCP); 48h FCCP followed by 24h FCCP and FAT (FCCP+FAT). Cells were harvested for experiments on day 7.

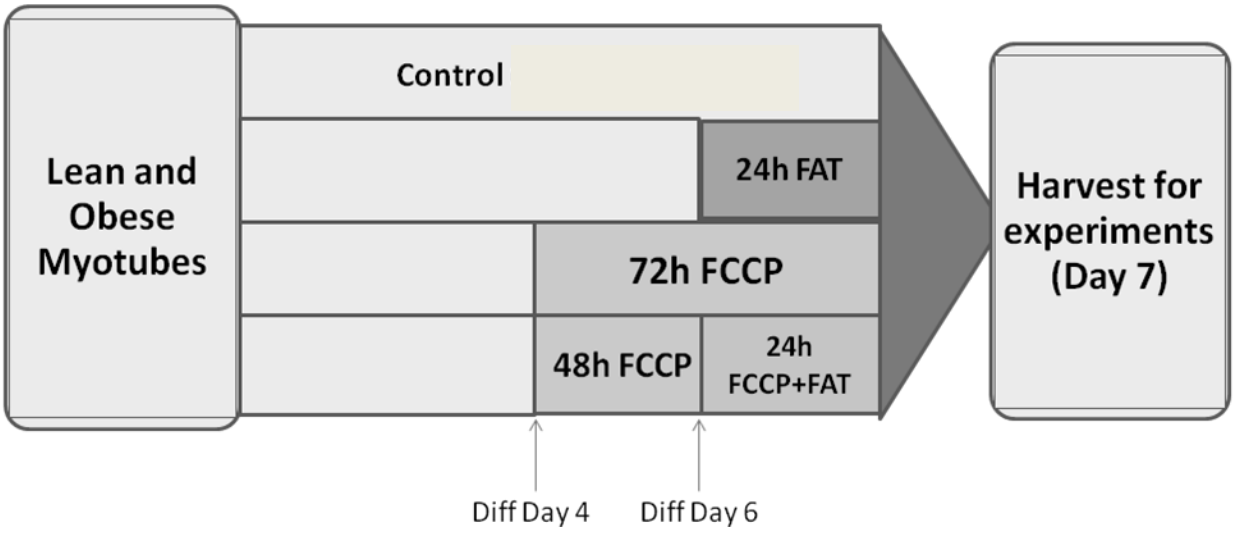


Fig. 3.2 Representative Western blot of OXPHOS protein content in response to differentiation media supplemented with 2% horse serum (Control), 100 µM of 1:1 oleate:palmitate (FAT), 72h 400 nM FCCP, or 48h FCCP + 24h combined FCCP and 1:1 oleate:palmitate. The OXPHOS antibody cocktail contained antibodies against the following subunits: Complex I subunit NDUFB8 (I), Complex II subunit 30kDa (II), Complex III subunit Core 2 (III), Complex IV subunit II, and ATP synthase subunit alpha (V). Complex IV subunit II could not be detected consistently in all of the samples; therefore, data for this protein was not analyzed.

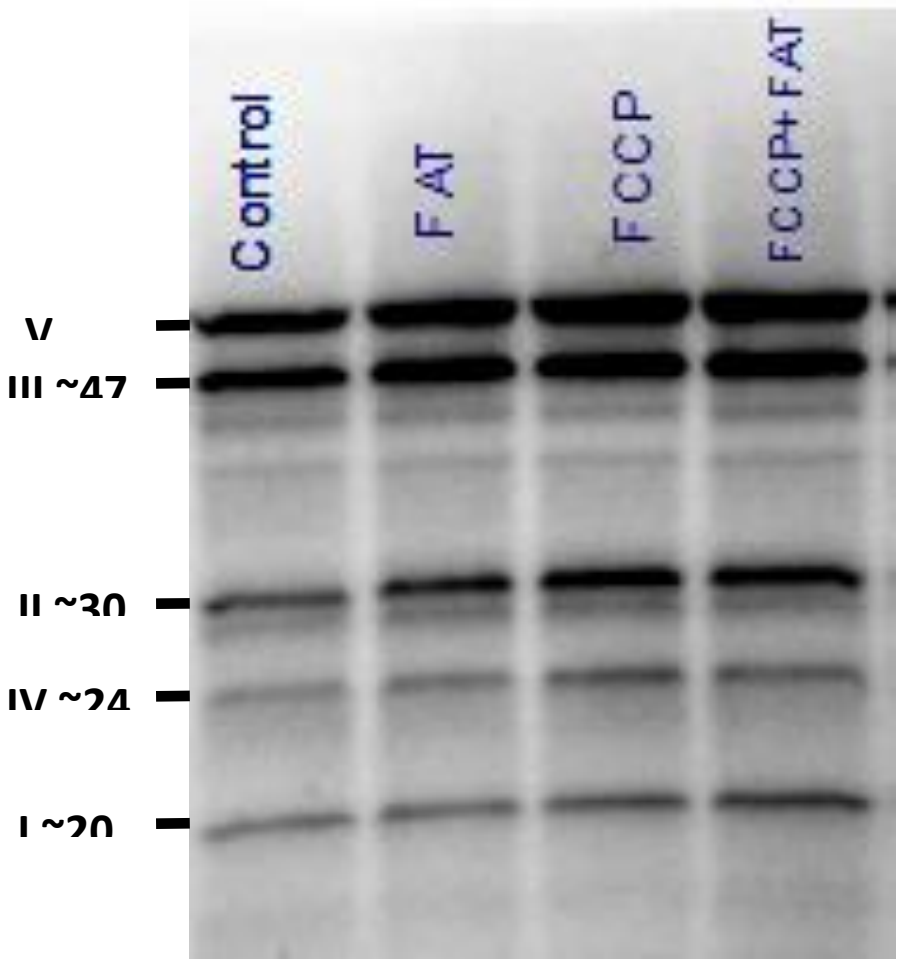


Fig. 3.3 Complete oleate oxidation ($^{14}CO_2$ production from oleate) in myotubes in response to 72h FCCP incubation.

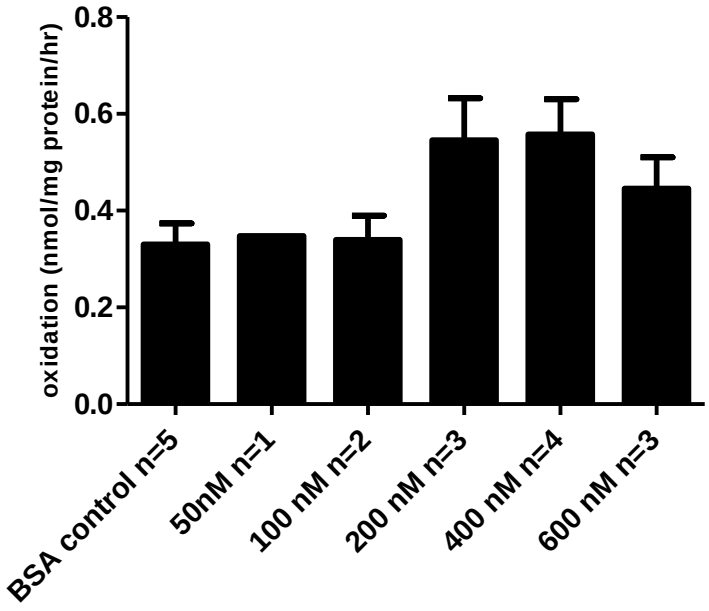
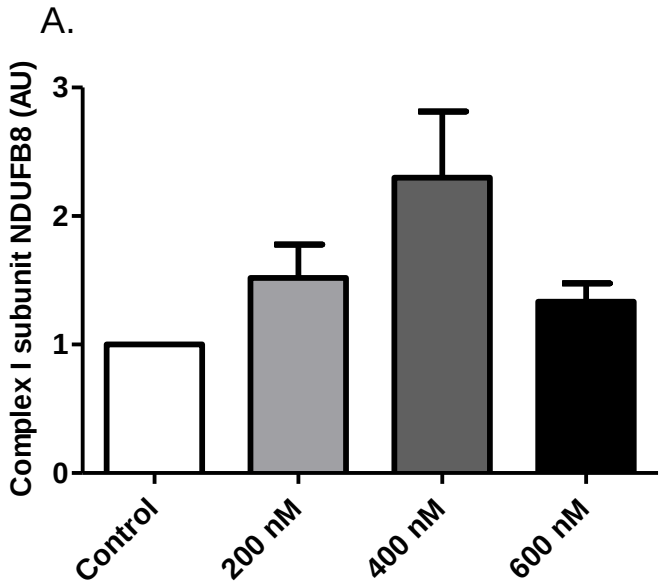
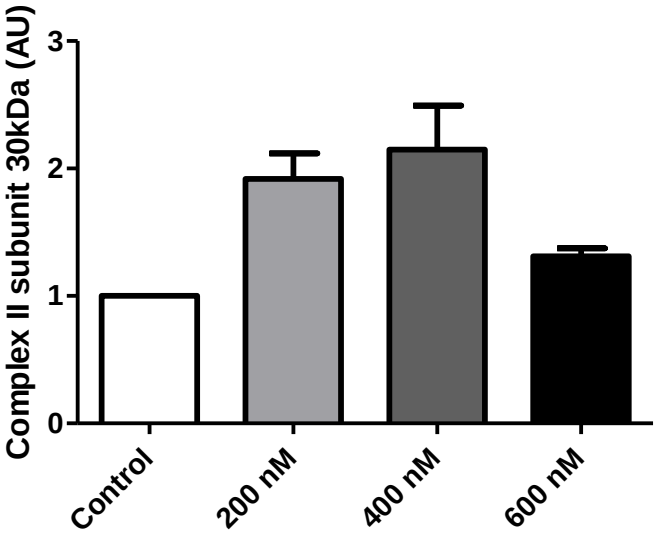


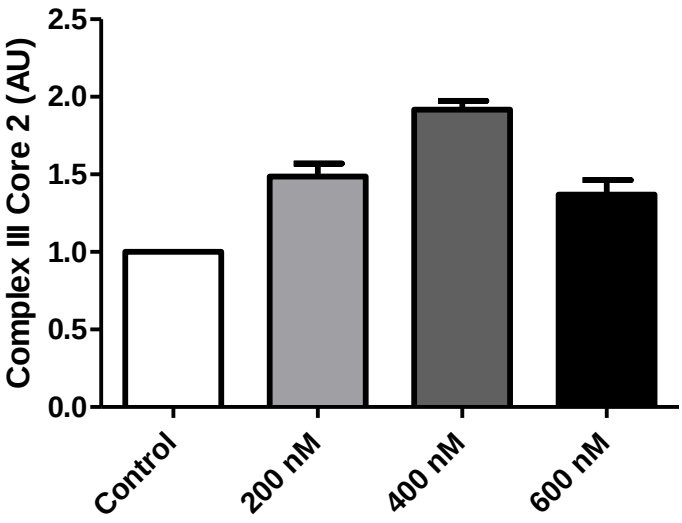
Fig. 3.4 Protein content of Complex I subunit NDUFB8 (A), Complex II subunit 30kDa (B), Complex III subunit Core 2 (C), and ATP synthase subunit alpha (D) was measured in response to 72h FCCP (n=2), and COXIV protein content (E) and citrate synthase enzyme activity (F) were measured in 1 lean individual. Protein content was expressed as fold-change in reference to the protein content of the respective control condition (control was set at 1 arbitrary unit), and citrate synthase activity was measured in umol/mg protein/min.



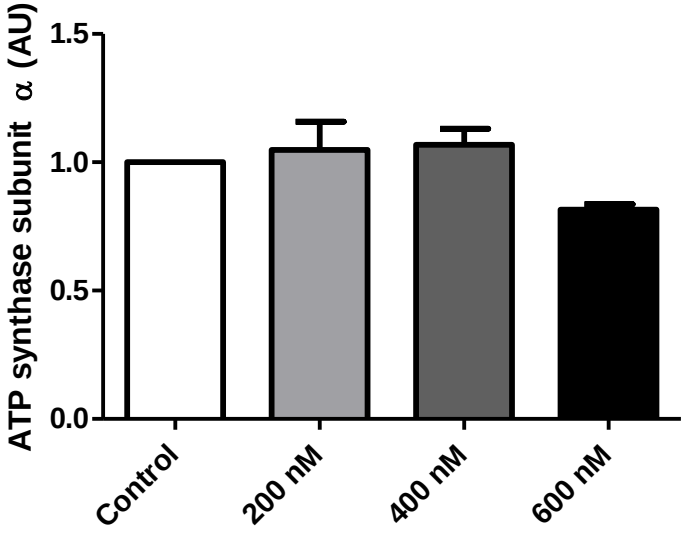
B.



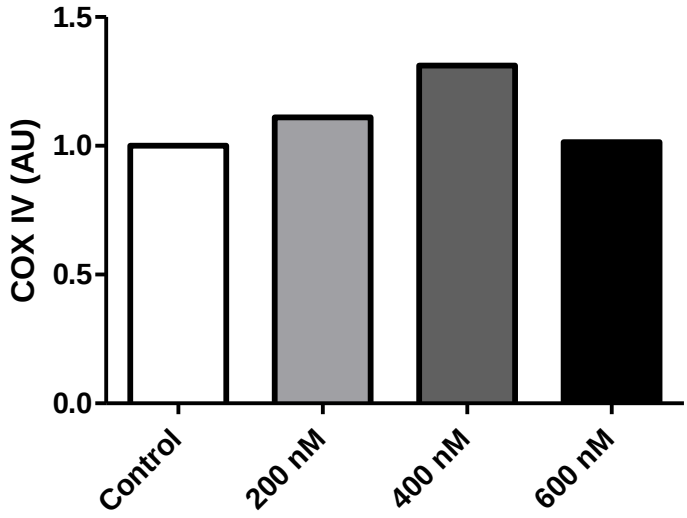
C.


Complex III Core 2 (AU)
2.5
2.0
1.5
1.0
0.5
0.0
Control
200 nM
400 nM
600 nM

D.


ATP synthase subunit α (AU)
1.5
1.0
0.5
0.0
Control
200 nM
400 nM
600 nM

E.



F.

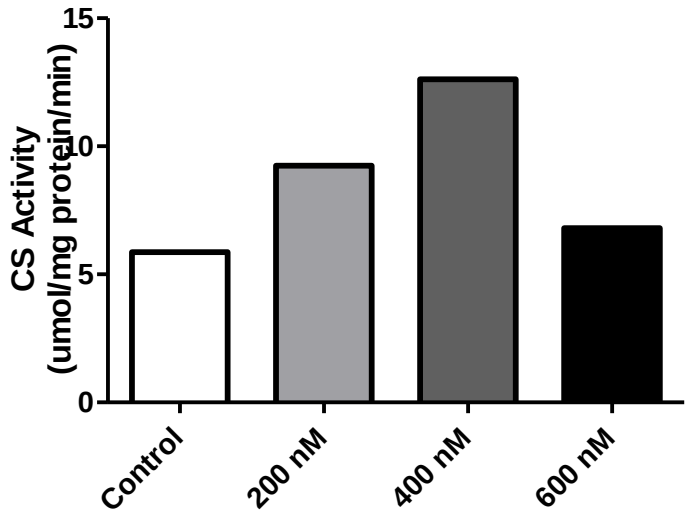
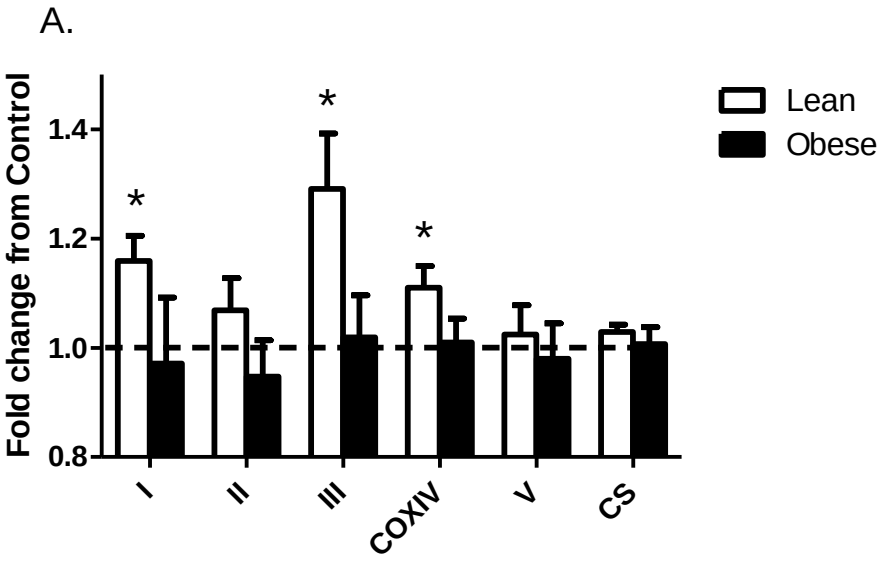
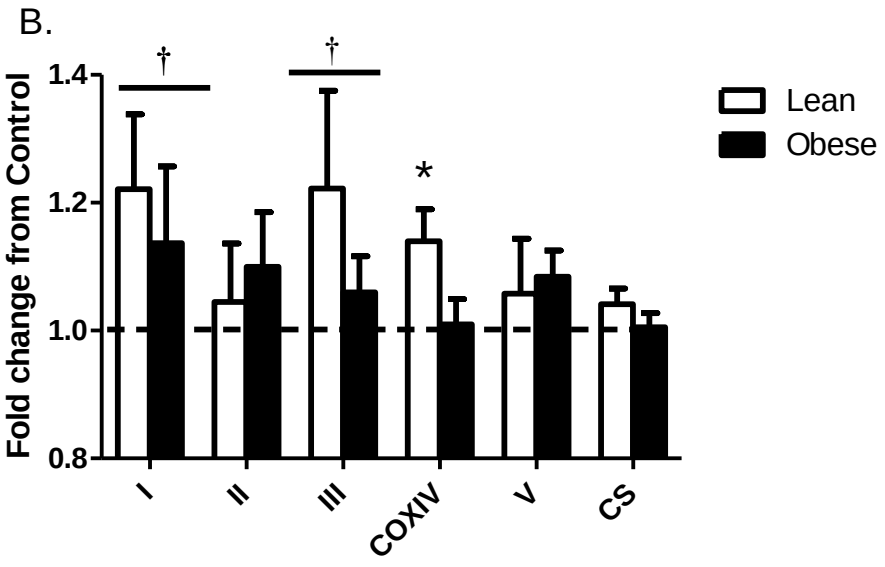

CS Activity
(umol/mg protein/min)
15
10
5
0
Control
200 nM
400 nM
600 nM

Fig. 3.5 Relative changes (post-treatment divided by pre-treatment) in content of Complex I subunit NDUFB8 (I), Complex II subunit 30kDa (II), Complex III subunit Core 2 (III), cytochrome c oxidase subunit IV (COX IV), ATP synthase subunit alpha (V), and citrate synthase (CS) activity in response to 24h incubation with 100 µM of 1:1 oleate:palmitate (FAT) (A), 72h 400 nM FCCP (B), or 48h FCCP + 24h combined FCCP and 1:1 oleate:palmitate (C). *Treatment effect for lean only; [I]Treatment effect for both lean and obese.




B.
†
†
*
1.4
1.2
1.0
0.8
Fold change from Control
I
II
III
COXIV
V
CS
Lean
Obese

C.

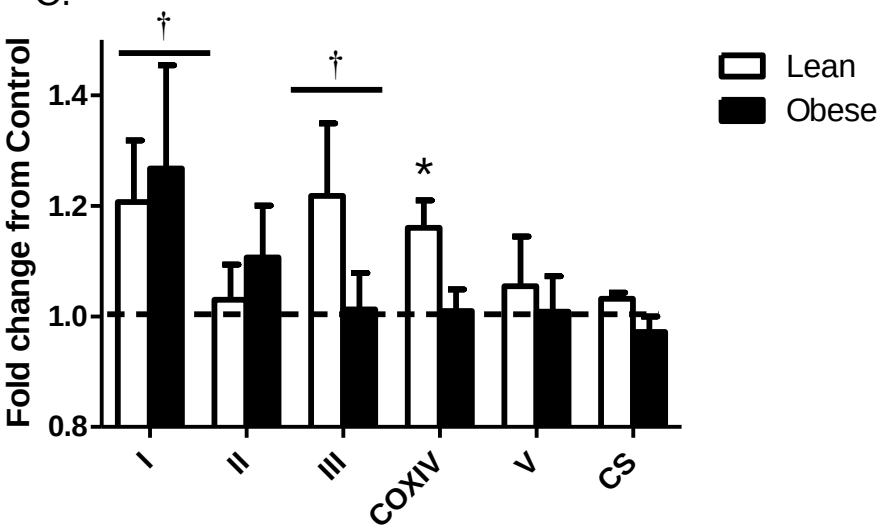
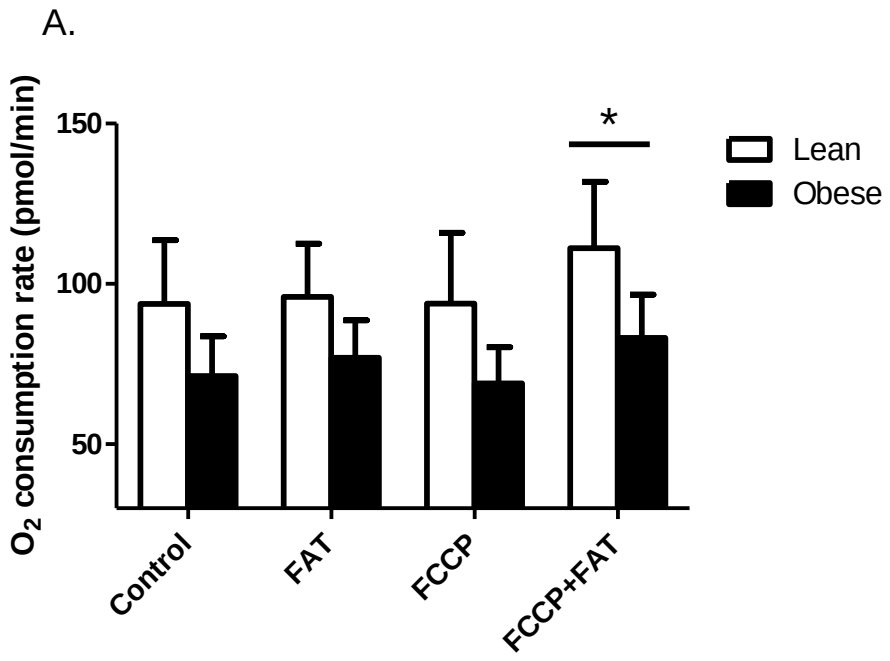
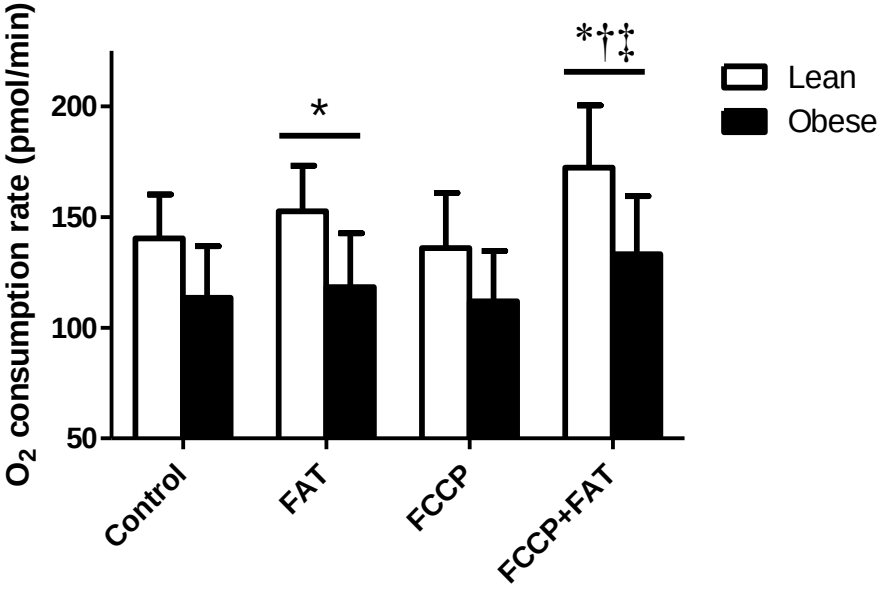

†
†
*
Fold change from Control
1.4
1.2
1.0
0.8
I
II
III
COXIV
V
CS
Lean
Obese

Fig. 3.6 Substrate-only (A) and 100 µM DNP-stimulated (B) oxygen consumption in the presence of 1 mM pyruvate and 200 µM 1:1 oleate:palmitate in myotubes from lean and obese donors incubated with control media (Control), 24h 100 µM 1:1 oleate:palmitate (FAT), 72h 400 nM FCCP (FCCP), or 48h FCCP + 24h combined FCCP and FAT (FCCP+FAT). *Significantly different from control; [I]Significantly different from FAT only; [ǂ]Significantly different from FCCP only.



B.


O_2 consumption rate (pmol/min)
200
150
100
50
*
*†‡
Control
FAT
FCCP
FCCP+FAT
Lean
Obese

CHAPTER 4: SUMMARY OF FINDINGS

This dissertation examined the effects of increased lipid load and aerobic exercise training on skeletal muscle metabolic flexibility in lean and obese individuals using *in vivo* (study 1) and cell culture (study 2) models. Our primary findings were 1) obese individuals have a blunted response to a 3-day 70% fat diet in terms of increasing skeletal muscle lipid oxidation, which was rescued by 10 days of aerobic exercise training and 2) similar to the *in vivo* high-fat diet (HFD), myotubes cultured from obese individuals displayed a similar inability to respond to increased lipid presence (24h lipid incubation) in terms of increasing mitochondrial protein content, which was not fully ameliorated by accelerating substrate utilization via mitochondrial uncoupling. Together, this suggests that the metabolic inflexibility to high lipid loads in obese individuals is persistent, and while *in vivo* exercise training is effective for rescuing this inflexibility to lipid, the mechanism for this response is complex and likely involves other factors besides increasing mitochondrial respiration.

As hypothesized in study 1, 3d of an isocaloric 70% fat diet increased skeletal muscle fatty acid oxidation (FAO) in lean, but not obese individuals (Fig. 2.2). This occurred in the absence of increases in electron transport chain protein content and activities of CS and HAD (Fig. 2.3A-B), indicating that perhaps improvements in substrate switching and/or mitochondrial function are responsible for the increased lipid oxidation. In particular, inactivation of the pyruvate dehydrogenase complex (PDC) via increased pyruvate dehydrogenase kinase 4 (PDK4) content and/or activity could be a possible mechanism for high-fat diet-induced increases in lipid oxidation, as both content and activity increase after 1d of a high-fat diet in lean individuals (9), and lean, but not obese individuals increase PDK4 mRNA content in response to a 5-day high-fat diet (3). Although we did not have sufficient tissue to measure PDK4 content and/or activity,

future research on metabolic flexibility in response to lipid should investigate this mechanism, as regulation of PDC could be instrumental in the adaptation to high-fat and/or high-carbohydrate diets in lean healthy individuals.

In study 2, short-term (24h) lipid incubation in cell culture showed a similar impairment in metabolic flexibility in myotubes from obese donors, as lean but not obese individuals increased protein content of Complex I, Complex III, and COX IV from the electron transport chain (Fig. 3.5A). Greater lipid presence in skeletal muscle activates the transcription of genes to increase oxidative capacity and enhance electron transfer efficiency in the mitochondria. Complex I, Ill, and COX IV may be particularly important for forming a "supramolecular assembly" that increases electron transfer efficiency in the mitochondria. Therefore, the cultured myotubes from the lean may increase protein content of these enzymes, and perhaps oxidative phosphorylation capacity in response to high lipid availability. However, the myotubes from obese individuals do not increase OXPHOS protein content in response to lipid incubation, mirroring the metabolic inflexibility we observed with our *in vivo* HFD in study 1. Much like our previous research in cultured myotubes investigating mitochondrial DNA content (4) and CD36 content (1), the current findings further indicate that obese individuals have a persistent impairment in response to lipid incubation in cell culture, suggesting dysregulation of many aspects of mitochondrial content and function with obesity.

In study 1, we investigated whether 10 days of aerobic exercise training could restore metabolic flexibility in response to a high-fat diet (HFD) in obese individuals. Obese subjects increased skeletal muscle FAO to the same extent as their lean counterparts in response to the high-fat diet while exercise training (Fig. 2.2), indicating that obese skeletal muscle has the

ability to increase FAO in response to aerobic training as we have previously indicated (2, 6). Neither group significantly increased FAO in response to the HFD while exercise training, indicating that metabolic flexibility *per se*, or the ability to increase skeletal muscle FAO after consuming a HFD, was not improved with exercise training. However, exercise training increased FAO equivalent to or beyond the level seen in response to the HFD alone, suggesting that a high absolute capacity for FAO can help obese skeletal muscle adjust to increased dietary lipid and minimize positive lipid balance.

We also observed increased citrate synthase (CS) activity with exercise training (Fig. 2.3A), similar to previous short-term exercise training studies (8, 12), and a tendency for beta-hydroxyacyl CoA dehydrogenase (HAD) activity to increase with exercise (Fig. 2.3B). However, the relatively modest increase in enzyme activity with exercise (19% increase in CS activity in the lean individuals) likely does not fully explain the more robust increase in overall FAO we observed in response to the combined diet and exercise intervention (55% increase in lean individuals). Therefore, it is likely that mitochondrial function also improved with exercise training. Other studies have suggested that the mitochondria of obese skeletal muscle has a deficit in NADH oxidase activity in the electron transport chain, perhaps promoting the accumulation of NADH (10), and does not increase mitochondrial DNA content to the same extent as lean individuals in response to 4 mo of exercise training (7). However, our findings do not suggest a lean vs. obese difference in terms of the ability to increase skeletal muscle FAO in response to short-term exercise training combined with a HFD, further supporting our hypothesis that obese skeletal muscle can respond to aerobic exercise training and improve oxidative capacity.

To determine whether speeding up mitochondrial respiration rate in cell culture could mimic *in vivo* exercise training and increase mitochondrial content, we incubated myotubes from lean and obese donors with the chemical uncoupler FCCP for 72h in study 2. However, obese did not appear to respond to FCCP incubation as robustly as the lean did, as the obese increased some, but not all, of the mitochondrial enzyme proteins we measured (Fig. 3.5B). We based our FCCP incubation methods on the findings from Rohas and colleagues, who incubated 10T ½ fibroblasts with FCCP for 72h and observed increased transcription of PGC-1 and downstream mitochondrial genes, concomitant with increased AMPK activation and intracellular Ca^{2+} levels (11). We did not measure AMPK activity, AMP:ATP ratios, or intracellular Ca^{2+} levels in the myotubes to assess the degree to which we were activating these pathways. Therefore, while increasing substrate utilization via FCCP incubation did not fully mimic *in vivo* exercise training, it did lead to increases in some mitochondrial proteins in obese individuals. Further research of the multiple pathways involved in exercise-induced increases in mitochondrial content will help clarify the mechanisms by which the obese increase skeletal muscle FAO in response to exercise in vivo.

Although 72h FCCP incubation increased protein content of Complex I, Complex III, and COX IV enzymes in lean HSkMC (Fig. 3.5B-C), 10d aerobic exercise training *in vivo* did not increase protein content of these enzymes (Fig. 2.4A-D). Furthermore, citrate synthase activity increased with 7d exercise, and 10d exercise combined with 3d high-fat diet in study 1 (Fig. 2.3A), but did not change with in vitro FCCP or FCCP+FAT incubation in study 2 (Fig. 3.5 B-C). Although both interventions increase cell respiration, incubation with FCCP *in vitro* may involve a chronic depolarization of the mitochondrial membrane for the 72h period. This may provide a greater stimulus to the electron transport chain enzymes that generate the proton

gradient across the mitochondrial membrane than the exercise training protocol employed in study 1, which involved 10 1h sessions of exercise interspersed by rest periods. While FCCP accelerates cell respiration within the mitochondria, the effects of exercise training on skeletal muscle are multifaceted, and increasing mitochondrial respiration is only one part of the overall effect. Muscle contraction-induced signaling, increases in skeletal muscle blood flow, and changes in the muscle's hormonal environment can all contribute to the increased skeletal muscle FAO capacity we found in study 1. Even at the molecular level, skeletal muscle can increase skeletal muscle FAO capacity via a variety of mechanisms besides increasing mitochondrial content, such as allowing greater transport of fatty acids into the mitochondria by increasing the lipid transport capacity of carnitine palmitoyltransferase-1 (CPT-1) (5). Therefore, accelerating substrate turnover in the electron transport chain in HSkMC *in vitro* had different effects than *in vivo* exercise training did on whole skeletal muscle.

In study 2, we assessed whether combined lipid and FCCP incubation could rescue the lipid-induced metabolic inflexibility we observe in cultured myotubes from obese individuals, similar to the *in vivo* exercise training and HFD in study 1. Although obese cells did not increase all of the mitochondrial proteins to the extent of the lean subjects (Fig. 3.5C), the combined FCCP+FAT incubation did increase maximally stimulated respiration rate in intact myotubes over control, FAT, and FCCP conditions for both lean and obese individuals (Fig. 3.6B), which was not in accordance with the mitochondrial content responses we observed. Since we measured respiration rate in intact cells, changes could be influenced by factors such as transport into the cell and/or mitochondria, shifts in relative substrate utilization, substrate flux through the electron transport chain, or oxidation of endogenous substrates. Respiration experiments in

which we isolate the mitochondria, or use inhibitors for ETC enzymes or substrates, could help elucidate potential mechanisms.

In conclusion, the findings indicate that *in vivo* the skeletal muscle of obese individuals does not adjust to increased lipid loads in terms of increasing lipid oxidation. In cell culture, myotubes from obese individuals do not increase mitochondrial protein in response to lipid incubation to the same extent as their lean counterparts, indicating a persistent impairment with obesity. Short-term exercise training rescues the response to a HFD in obese individuals; however, increasing substrate turnover in an *in vitro* cell culture model does not completely restore lipid-induced metabolic flexibility, suggesting that other factors contribute to the effects of exercise training on the response to increased lipid loads in vivo and should be further examined.

References

1. **Bell JA, Reed MA, Consitt LA, Martin OJ, Haynie KR, Hulver MW, Muoio DM, and Dohm GL**. Lipid partitioning, incomplete fatty acid oxidation, and insulin signal transduction in primary human muscle cells: effects of severe obesity, fatty acid incubation, and fatty acid translocase/CD36 overexpression. *The Journal of clinical endocrinology and metabolism* 95: 3400-3410, 2010.
2. **Berggren JR, Boyle KE, Chapman WH, and Houmard JA**. Skeletal muscle lipid oxidation and obesity: influence of weight loss and exercise. *Am J Physiol Endocrinol Metab* 294: E726-732, 2008.
3. **Boyle KE, Canham JP, Consitt LA, Zheng D, Koves TR, Gavin TP, Holbert D, Neufer PD, Ilkayeva O, Muoio DM, and Houmard JA**. A high-fat diet elicits differential responses in genes coordinating oxidative metabolism in skeletal muscle of lean and obese individuals. *The Journal of clinical endocrinology and metabolism* 96: 775-781, 2011.
4. **Boyle KE, Zheng D, Anderson EJ, Neufer PD, and Houmard JA**. Mitochondrial lipid oxidation is impaired in cultured myotubes from obese humans. *International journal of obesity* 2011.
5. **Bruce CR, Thrush AB, Mertz VA, Bezaire V, Chabowski A, Heigenhauser GJ, and Dyck DJ**. 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-E107, 2006.
6. **Cortright RN, Sandhoff KM, Basilio JL, Berggren JR, Hickner RC, Hulver MW, Dohm GL, and Houmard JA**. Skeletal muscle fat oxidation is increased in African-American and white women after 10 days of endurance exercise training. *Obesity* 14: 1201-1210, 2006.
7. **Menshikova EV, Ritov VB, Ferrell RE, Azuma K, Goodpaster BH, and Kelley DE**. Characteristics of skeletal muscle mitochondrial biogenesis induced by moderate-intensity exercise and weight loss in obesity. *J Appl Physiol* 103: 21-27, 2007.
8. **Perry CG, Lally J, Holloway GP, Heigenhauser GJ, Bonen A, and Spriet LL**. Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle. *J Physiol* 588: 4795-4810, 2010.
9. **Peters SJ, Harris RA, Wu P, Pehleman TL, Heigenhauser GJ, and Spriet LL**. Human skeletal muscle PDH kinase activity and isoform expression during a 3-day high-fat/low-carbohydrate diet. *Am J Physiol Endocrinol Metab* 281: E1151-1158, 2001.
10. **Ritov VB, Menshikova EV, Azuma K, Wood R, Toledo FG, Goodpaster BH, Ruderman NB, and Kelley DE**. Deficiency of electron transport chain in human skeletal muscle mitochondria in type 2 diabetes mellitus and obesity. *Am J Physiol Endocrinol Metab* 298: E49-58, 2010.
11. **Rohas LM, St-Pierre J, Uldry M, Jager S, Handschin C, and Spiegelman BM**. A fundamental system of cellular energy homeostasis regulated by PGC-1alpha. *Proc Natl Acad Sci U S A* 104: 7933-7938, 2007.
12. **Spina RJ, Chi MM, Hopkins MG, Nemeth PM, Lowry OH, and Holloszy JO**. Mitochondrial enzymes increase in muscle in response to 7-10 days of cycle exercise. *J Appl Physiol* 80: 2250-2254, 1996.

APPENDIX: IRB APPROVAL

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
1L-09 Brody Medical Sciences Building· Mail Stop 682
600 Moye Boulevard · Greenville, NC 27834
Office **252-744-2914** · Fax **252-744-2284** · www.ecu.edu/irb

Notification of Continuing Review Approval

From: Biomedical IRB
To: Joseph Houmard
CC:

Gabriel Dubis
Date: 12/21/2011
Re: CR00000118
UMCIRB 06-0080
Lipid Metabolism in Obesity, Weight Loss and Exercise (2): Muscle Cell Studies

I am pleased to inform you that at the convened meeting on 12/21/2011 12:00 AM of the Biomedical IRB , this research study underwent a continuing review and the committee voted to approve the study. Approval of the study and the consent form(s) is for the period of 12/21/2011 to 12/20/2012.

The Biomedical IRB deemed this study Greater than Minimal Risk.

Changes to this approved research may not be initiated without UMCIRB review except when necessary to eliminate an apparent immediate hazard to the participant. All unanticipated problems involving risks to participants and others must be promptly reported to the UMCIRB. The investigator must submit a continuing review/closure application to the UMCIRB prior to the date of study expiration. The investigator must adhere to all reporting requirements for this study.

The approval includes the following items:

Name	Description	Modified	Version
Advertisments	Recruitment Documents/Scripts	12/8/2011 7:48 PM	0.01
Full Protocol	Study Protocol or Grant Application	12/8/2011 7:36 PM	0.01
Protocol	Study Protocol or Grant Application	12/8/2011 7:18 PM	0.01
Version 4 exercise training.doc	Consent Forms	12/8/2011 8:28 PM	0.01
Version 4 cell culture studies.doc	Consent Forms	12/8/2011 8:27 PM	0.01
Version 4 HFD and exercise.doc	Consent Forms	12/8/2011 8:28 PM	0.01
Version 4 HFD and weight loss.doc	Consent Forms	12/8/2011 8:28 PM	0.01
Version 4 HFD.doc	Consent Forms	12/8/2011 8:28 PM	0.01

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study: