THE EFFECT OF CONTRACTILE ACTIVITY AND SUBSTRATE CHALLENGES ON METABOLIC FLEXIBILITY IN HUMAN PRIMARY MYOTUBES

by

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May, 2013

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The skeletal muscle of severely obese individuals (BMI $\geq$ 40 kg/m^2) is characterized by a depressed ability to oxidize fatty acids and a failure to upregulate fatty acid oxidation (FAO) in response to increased lipid availability, both of which may contribute to a positive lipid balance and weight gain. The inability to switch from predominately glucose oxidation to FAO in the presence of a lipid challenge, such as a high-fat diet (HFD), is part of a defect in obesity known as *metabolic inflexibility*. In severe obesity, ten consecutive days of aerobic training (short-term training) partially restores metabolic flexibility by increasing the ability of skeletal muscle to oxidize fatty acids. The purpose of this dissertation was to determine a) whether contractile activity in human primary skeletal muscle cell culture (HSkMC) via electrical stimulation provides a model to investigate the mechanisms underlying the ability of short-term aerobic training to normalize FAO and provide some degree of metabolic flexibility in severely obese individuals and b) whether the *metabolic inflexibility* present in obesity is unique to a lipid challenge such as a HFD or whether obese individuals are also metabolically inflexible when

challenged with carbohydrates (CHO) such as galactose or pyruvate. The hypotheses were that 1) HSkMC would provide a model to investigate how *in vivo* contractile activity increases FAO and markers of mitochondrial content in response to short-term aerobic training and 2) HSkMC from severely obese individuals would be *metabolically flexible* in response to galactose or pyruvate but would remain *metabolically inflexible* to a lipid challenge. The results contained herein suggest that contractile activity in HSkMC for 48 h may be capable of mimicking some of the short-term training effects such as increased FAO; however, a longer electrical stimulation period is warranted to initiate changes in mitochondrial content and oxidative capacity. Although comparisons between lean and obese were not made in this study, the results and recommendations of the present investigation have laid the groundwork for future studies to investigate the effect of electrical stimulation on HSkMC established from severely obese individuals.

In the second study of this investigation, HSkMC established from severely obese individuals were found to be metabolically flexible in response to 24 h incubations with lipid, galactose, or pyruvate as evidenced by an increase in state 3 palmitoyl-carnitine malate (PCM_3) and FCCP stimulated respiration in response to all three substrates. Citrate synthase activity and OXPHOS protein content also increased whereas glucose utilization decreased in response to substrate challenges in both lean and obese groups. The volunteers in the second study were young and likely not far into the progression of metabolic disease, which may explain why metabolic flexibility remained intact in this population. Although limited by the inability to fully recapitulate true physiological conditions, human primary skeletal muscle culture is a novel tool for investigations into the mechanisms underlying metabolic inflexibility in obesity.

THE EFFECT OF CONTRACTILE ACTIVITY AND SUBSTRATE CHALLENGES ON METABOLIC FLEXIBILITY IN HUMAN PRIMARY MYOTUBES

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

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ACKNOWLEDGEMENTS

I would first like to thank my PhD advisor, Dr. Joseph A. Houmard, for helping me to learn more about obesity and metabolism than I ever would have been able to learn on my own. I would also like to thank the members of my committee, Drs. Ethan J. Anderson, David A. Brown, and Saame R. Shaikh for providing the advice and direction necessary to get me and keep me on track to graduate. You have taught me many lessons that I will be able to apply to my future career. I would like to thank Drs. Jeffrey J. Brault and Carol A. Witczak for their scientific support and continued encouragement. I hope you both know that you are doing a fantastic job and I truly appreciate everything you have done for me.

I want to thank Jill Maples for being the best lab mate a PhD student could ever ask for. No one works harder than you and I wish you the very best in the future. To Gina Battaglia for accompanying me for an endless number of hours spent in the cell culture room. It was great not being the only one in the Brody School of Medicine every weekend. Joe Pierce for all the help with making electrical stimulation videos, for your sense of humor, and jokes. You're a great all around guy. Kelsey Fisher-Wellman for introducing me to the galactose literature and partnering up in data collection. I would not have finished this degree program without your assistance; I owe you a debt of gratitude. To Kathleen Gavin, my dearest friend and fellow scientist. I never and I mean never would have finished this degree without your continued support. I consider myself to be so lucky to have found you. Words cannot describe what you mean to me.

I would like to thank my family, who has not just supported me through my doctoral studies but have supported me throughout my entire life. To my brother Ryan, for his willingness to always be there when I need him whether that means traveling from Wisconsin to Texas, Iowa, North Carolina, or Colorado, I know I can count on you. To my sister, Karen, and

brother-n-law, Mike, you have always been role models and mentors to me as well as being the parents to three wonderful children, Ava, Finn, and Liam, who remind me of what life is truly about. Finally, I would like to thank my parents for always believing in me, supporting me, and encouraging me. Your love and support has helped shape the person who I am today and for that I am truly grateful.

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LIST OF ABBREVIATIONS

AMPK:	5’ adenosine monophosphate-activated protein kinase
ADP:	adenosine diphosphate
AKT:	protein Kinase B
ANOVA:	analysis of variance
ATP:	adenosine triphosphate
AU:	arbitrary units
β-HAD:	beta-hydroxyacyl coenzyme A dehydrogenase
BCA:	bicinchoninic acid
BMI:	body mass index
BSA:	bovine serum albumin
CaMKII:	calcium/calmodulin-dependent protein kinase II
CHO:	carbohydrate
CO_2:	carbon dioxide
CoA:	coenzyme A
COX-IV:	cytochrome c oxidase, isoform IV
CS:	citrate synthase activity
Cyt C:	cytochrome C
D:	ADP
DMEM:	Dulbecco’s Modified Eagle Medium
ECL:	enhanced chemiluminescence
ECM-gel	extracellular matrix gel

EDTA:	ethylenediaminetetraacetic acid
eEF2:	eukaryotic elongation factor 2
EGTA:	ethylene glycol tetraacetic acid
FAO:	fatty acid oxidation
FBS:	fetal bovine serum
FCCP:	carbonyl cyanide p-(triflurometoxy) phenyl hydrazone
FFM:	fat-free mass
G:	glutamate
Gal:	galactose
Gent:	gentamicin
GM:	glutamate-malate
HbA1c:	glycosylated hemoglobin
HEPES:	4- (2-hydroethyl)-1-piperazineethenesulfonic acid
HFD:	high-fat diet
HOMA-IR:	homeostasis model of assessment-insulin resistance
HSkMC:	primary human skeletal muscle cells raised in culture
Hz:	hertz
IGF-1	insulin like growth factor-1
JO_2:	oxygen consumption
M:	malate
MCT:	monocarboxylate transporter
MHC:	myosin heavy chain
mRNA:	messenger ribonucleic acid

ms:	milliseconds
mtDNA	mitochondrial DNA
mTOR:	mammalian target of rapamycin
NHANES:	National Health and Nutrition Examination Survey
O/P:	oleate/palmitate
OXPHOS:	oxidative phosphorylation
p70-S6:	70 kDa ribosomal protein S6 kinase 1
PBS:	phosphate buffered saline
PC:	palmitoyl-carnitine
PCM_3:	state 3 palmitoyl-carnitine malate
PGC-1α:	peroxisome proliferator-activated receptor γ co-activator 1 alpha
P/S:	penicillin/streptomycin
PVDF:	polyvinylidene fluoride
Pyr:	pyruvate
RER:	respiratory exchange ratio
RMR:	resting metabolic rate
RQ:	respiratory quotient
S:	succinate
S_3:	state 3 succinate
S_4:	state 4 succinate
SDH:	succinate dehydrogenase
SDS:	sodium dodecyl sulfate
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis

sec:	seconds
SEM:	standard error of the mean
Stim:	electrical stimulation
TCA:	tricarboxylic acid
TFAM:	mitochondrial transcription factor A
V:	volts
VO_2max:	maximal oxygen consumption
VO_2peak:	peak rate of oxygen consumption
4E-BP1:	eukaryotic translation initiation factor 4E-binding protein 1

CHAPTER 1: LITERATURE REVIEW

Prevalence and Etiology of Obesity

The prevalence of obesity, which is defined as having a body mass index (BMI) ≥ 30 kg/m^2, is increasing at an alarming rate in Westernized societies and developing countries (146). In the United States the prevalence of obesity during the last 40 yrs among adults has increased from 11.9% to 35.5% in men and from 16.6% to 35.8% in women and severe obesity (BMI ≥ 40 kg/m^2) has increased from 0.6% to 4.4% and 2.1% to 8.2% in men and women respectively (53, 54).

In and of itself, obesity may not directly cause metabolic disease (157, 185) but is permissive for the development of several chronic diseases including heart disease, type 2 diabetes, hypertension, stroke, and certain types of cancer (206). The risk of developing such chronic diseases is positively associated with the degree of adiposity with a linear increase in morbidity and mortality with body mass indexes ≥ 25 kg/m^2 (201). Individuals with severe obesity are at the greatest risk and can anticipate a reduction of 8-10 yrs in life expectancy compared to their lean counterparts (201).

Obesity is a complex multifaceted condition whose etiology is not fully understood, however, a central tenet of obesity is chronic energy imbalance, i.e., energy intake that exceeds energy expenditure over a prolonged period of time (75). The *two primary* contributors to the positive energy imbalance present in the development of obesity are thought to be 1) decreased physical activity (81) and 2) excess caloric consumption (174, 175). Our lifestyles have changed considerably over the recent past: there has been a substantial decrease in the number of occupations requiring moderate amounts of physical activity (33), the amount of physical activity in our day-to-day lives has largely been engineered out of our environment through

technological advances, and access to inexpensive energy-dense foods is abundant, and convenient (79, 139, 140). Although energy imbalance is the generally accepted cause of obesity, some in the field have challenged this paradigm, suggesting there are instances in which energetic balance is achieved whereas macronutrient balance is not (5, 52, 80). In this paradigm, the *third* contributor to the development of obesity is macronutrient balance, which can be summarized in the simple equation: Intake – Oxidation = Balance. Carbohydrate and fat balances are intricately tied to one another, the preferential oxidation of carbohydrate or fat will contribute to a positive imbalance of the reciprocal macronutrient (80). In theory, an overreliance on CHO oxidation is thought to result in a short-term positive fat balance. Over time, these short-term positive fat balances may accumulate and translate into weight gain. At the whole body level, the contribution of carbohydrate and fat can be calculated through the ratio of expired gases (O_2 to CO_2) known as the respiratory exchange ratio (RER) or respiratory quotient (RQ). The RER ranges from 0.7 to 1.0 with 0.7, 0.85, and 1.0 indicating approximately 100/0, 50/50, and 0/100 percentages of fat and carbohydrate utilization, respectively. Zurlo et al (211) was one of the first investigators to demonstrate that an elevated fasting RQ was predictive of future weight gain in the Pima Indian population of Arizona. Since then, other investigators have demonstrated that an elevated RQ is predictive of future weight gain in non-obese women (118, 119) and in a large cohort of men with ages ranging from 18-98 (164). Although these short-term macronutrient imbalances may seem trivial, they are in line with evidence indicating that obesity is the result of relatively small daily positive energy imbalances with estimates ranging from 100 kcal/d (81) to 110-165 kcal/d (195). The question must then be asked, if small macronutrient imbalances contribute to weight gain, what steps can be taken to correct them.

Skeletal Muscle Fatty Acid Oxidation

Fat free mass (FFM) is the largest contributor to resting metabolic rate (RMR). Although skeletal muscle is not considered to be one of the most metabolically active tissues, by mass, it makes up to ~40-50% of FFM (58, 84) and therefore represents a substantial portion (~20-30%) of total oxygen consumption (210). For a positive fat balance to occur, fat storage must exceed fat oxidation. In our laboratory we have demonstrated that in severely obese individuals fatty acid oxidation (FAO) is reduced in muscle strips from rectus abdominus (88), muscle homogenates from the vastus lateralis (97), and whole body FAO using radiolabeled tracers (183). A reduction in the ability to oxidize fatty acids will in turn promote fat storage, which may contribute to a positive fat balance. Numerous investigators have shown that weight loss through dietary interventions drastically improves metabolic health but has little effect on the ability of previously obese individuals to oxidize fatty acids (5, 14, 94, 159, 183), leaving them prone to weight regain (5, 55, 118, 119, 191).

Our laboratory has also shown that 10 consecutive days of aerobic training rescues FAO by increasing skeletal muscle FAO in lean, obese, and previously obese individuals to the same extent (14), making exercise one of the few interventions to correct the impaired ability to oxidize fatty acids in the obese individual. On the other hand, the ability of the skeletal muscle of obese individuals to respond to aerobic exercise training has been questioned by some (39, 77). The transcriptional coactivator, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α) is responsible for coordinately upregulating the transcription of genes of oxidative metabolism (205). In lean, healthy individuals, PGC-1α mRNA transiently increases in response to aerobic training (144) whereas in obese insulin-resistant individuals this effect seems to be blunted (39). In a related study, PGC-1α mRNA and markers of

mitochondrial content have also been shown to be unresponsive to 6 wk of aerobic training in obese individuals (77) although not all data supports this notion (123).

Metabolic Flexibility

The skeletal muscle of obese individuals is also characterized by the inability to match substrate oxidation with nutrient availability, which may also contribute to a positive fat balance. In the late 1990's David Kelley's group coined the term "metabolic flexibility" to describe the ability of lean individuals to match substrate oxidation with substrate availability during times of fasting and insulin stimulation (94, 96). Obese individuals, on the other hand, are thought to be "metabolically inflexible" as they tend to remain overly reliant on CHO oxidation during fasting and less reliant during insulin stimulation. Our laboratory (10, 22, 23) and others (5, 182) have also found that obese individuals exhibit impaired "metabolic flexibility" in the face of a lipid challenge. For example, the imposition of a high fat diet increases PGC-1α mRNA, genes associated with lipid metabolism (22), and FAO (10) in lean, but not obese individuals. In addition, our laboratory has also demonstrated that human primary skeletal muscle cells (HSkMC) from obese individuals fail to upregulate the capacity to oxidize fatty acids in response to a 24 h lipid incubation (23), suggesting a genetic or epigenetic component to the etiology of obesity. Taken together, the inability to upregulate genes associated with oxidative metabolism and FAO in response to increased dietary fat or exercise may result in a positive lipid balance and leave the obese individual vulnerable to future weight gain.

Specific Aims and Hypotheses

To determine whether obese individuals are resistant to stimuli involved in increasing mitochondrial content and FAO, two primary aims have been developed, both of which were

performed in HSkMC. HSkMC provides an ideal experimental set up for studying muscle metabolism because it precludes the interfering actions of whole body, extramuscular effectors such as insulin, free fatty acids, and cytokines. In addition, severely obese individuals possess a significantly reduced oxidative capacity and as such a diminished exercise tolerance.

The purpose of the first aim of this investigation was to develop an *in vitro* model of short-term exercise training (contractile activity) to determine whether contractile activity is capable of increasing markers of mitochondrial content and FAO in myotubes derived from lean and obese donors to a similar extent. We hypothesized that *in vitro* contractile activity would provide a novel model to investigate the metabolic effects of short-term exercise training while minimizing many of the extramuscular effects and exercise intolerance inherent to the obese population.

The second aim of this investigation was to determine whether the obese individual remains metabolically inflexible in the presence of oxidizable substrates other than lipid. We hypothesize that metabolic flexibility will be conserved in severely obese individuals in the presence of oxidizable substrates other than lipid and that metabolic inflexibility in severe obesity is unique to lipid handling.

CHAPTER 2: CHRONIC ELECTRICAL STIMULATION OF HUMAN SKELETAL MUSCLE MYOTUBES AS A MODEL OF SHORT-TERM AEROBIC EXERCISE TRAINING

Abstract

The skeletal muscle of severely obese individuals is characterized by an inability to oxidize fatty acids. Ten consecutive days of aerobic training has been shown to reverse the decrement in fatty acid oxidation (FAO) to the point where there is no difference in FAO between lean and obese individuals. To gain a better understanding of how contractile activity increases FAO in skeletal muscle we sought to establish an *in vitro* model of exercise training. Human skeletal muscle cell cultures (HSkMC) from lean individuals and C2C12 skeletal muscle cells were intermittently electrically stimulated for 3 h/d for 3-4 d or continuously stimulated for 48 h according to protocols identified in the scientific literature. Fatty acid oxidation, oxygen consumption, and protein content of OXPHOS enzymes were determined in HSkMC, and glucose and lactate concentrations in the media were measured. Using the electrical stimulation parameters of 50 V 10 Hz (4 x 0.3 ms pulses delivered in 400 ms trains with 3.6 s recovery), a promising trend (1.3 fold increase in FAO, $P = 0.12$) was observed ($n = 3$); however, this trend was no longer evident (0.91 fold increase, $P = 0.59$) when increasing the sample size ($n = 5$). Using another intermittent protocol, we were unable to replicate the increase in COX IV protein observed by Uguccioni & Hood in either HSkMC ($n = 4$) or C2C12 myotubes ($n = 10$). The aminoglycoside antibiotic, gentamicin, present in our culture media has been shown to alter calcium handling and contractility in cardiac cells. We next sought to investigate whether gentamicin prevented electrically stimulated mitochondrial adaptations. The protocols of

Donnelly et al (n = 3), Uguccioni & Hood (n = 2), and Burch et al (n = 3) were unable to increase COX IV protein in C2C12 cells, however, the protocol of Pinheiro et al (n = 2) significantly increased ($P < 0.05$) COX IV protein in the presence of penicillin/streptomycin but not gentamicin. Additional experiments utilizing the Pinheiro et al protocol in HSkMC failed to increase COX IV or OXPHOS protein despite the absence of gentamicin from the culture media (n = 4). Continuously stimulating HSkMC for 48 h significantly increased FAO ($P < 0.05$) despite no change in OXPHOS protein or respiratory capacity in permeabilized myotubes. During the 48 h stimulation period, media was changed every 12 h and media lactate concentration significantly increased ($P < 0.05$) in the electrically stimulated cells at every time point, signaling an increase in glycolytic metabolism. The addition of lipid to the culture media during the 48 h stimulation period did not further enhance FAO beyond the presence of lipid alone ($P > 0.05$). We conclude that 1) efforts should be made to enhance HSkMC maturation and excitability in future electrical stimulation investigations, 2) antibiotic should not be included in the culture media during stimulation, 3) continuous stimulation of 48 h is preferable to short term intermittent stimulation (3 h/d, 3 d) in terms of increasing FAO, and 4) continuous stimulation periods greater than 48 h may be required to increase mitochondrial content and oxidative capacity to be able to truly recapitulate the full effects of short-term *in vivo* aerobic training.

Introduction

The skeletal muscle of severely obese (BMI > 40 kg/m^2) individuals is characterized by severe insulin resistance (59), reduced mitochondrial content (36, 85, 97), and a decreased reliance on fatty acids as a fuel source (97). The inability to oxidize fatty acids in severe obesity

is thought to be the result of reduced mitochondrial content and/or mitochondrial dysfunction (36, 85, 151, 152). Weight loss through caloric restriction has been shown to drastically increase insulin sensitivity (184) despite unchanged oxidative capacity. However, weight loss alone does not correct the impairment in fatty acid oxidation (FAO) observed in obesity, leaving formerly obese individuals prone to recidivism (14). A recent study by Battaglia et al. (10) highlighted the obese individual's vulnerability to a positive lipid balance as three days of a high-fat diet significantly increased FAO in lean individuals whereas the obese group experienced no change. Furthermore, when obese individuals undergo a short-term high-fat feeding, genes responsible for up-regulating lipid metabolism are insensitive (22), rendering the obese individual unresponsive to the lipid stimulus and at an increased risk of developing a positive lipid balance. Our laboratory has demonstrated that 10 consecutive days of aerobic training rescues FAO by increasing skeletal muscle FAO in lean, obese, and previously obese individuals to the same extent (14), making exercise one of the few interventions to correct the impaired ability to oxidize fatty acids in the obese individual. On the other hand, the ability of the skeletal muscle of obese individuals to respond to aerobic exercise training has been questioned by some (39, 77). The transcriptional coactivator, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α) is responsible for coordinately upregulating the transcription of genes of oxidative metabolism (205). In lean, healthy individuals, PGC-1α mRNA transiently increases in response to aerobic training (144) whereas in obese insulin-resistant individuals this effect seems to be blunted (39). In a related study, PGC-1α mRNA and markers of mitochondrial content have also been shown to be unresponsive to 6 wk of aerobic training in obese individuals (77) although not all data supports this notion (123).

Discovering the exercise-related mechanisms responsible for the improvement of fatty acid oxidation in obesity is of great importance in establishing and refining treatment options for severe obesity. Ideally, the effects of exercise on the skeletal muscle of obese individuals would be studied in *vivo*. However, there are a number of factors inherent to the obese subject that makes it difficult to study muscle metabolism including exercise intolerance, hyperinsulinemia, elevated plasma free fatty acids, and other endocrine and humoral factors. The phenotypic traits of skeletal muscle are retained in culture, making human skeletal muscle cell culture (HSkMC) a suitable method for studying obesity and other metabolic diseases (15, 23, 67). Furthermore, HSkMC provides a high throughput for studying the effects of inhibitors, the effect of various substrate incubations, and provides the ability to track muscle metabolism over time. Therefore, the purpose of this investigation was to optimize an electrical stimulation protocol in cultured cells capable of mimicking short-term *in-vivo* exercise training. In doing so, we would be able to study the effects of exercise in skeletal muscle cells of lean and severely obese individuals without the potential *in vivo* confounders inherent to the obese population.

Methods

Experimental Design. The purpose of this investigation was to optimize an electrical stimulation protocol in cultured cells capable of mimicking short-term *in-vivo* exercise training. There are a number of electrical stimulation protocols in the published literature, many of which have been performed in immortalized cell lines such as C2C12s (27, 28, 35, 42, 56, 89, 121, 129, 148, 181, 188, 190, 207) and L6 (208), chick (45), mouse (148), rabbit (103, 104) and rat primary cells (46, 48, 90, 128, 145, 154, 165, 172, 187, 190, 196) and recently in human primary skeletal myotubes (1, 26, 101, 107, 131). Electrical stimulation has been utilized to investigate a

broad spectrum of research topics including calcium handling (48, 101, 103, 190), fiber type transformations (28, 45, 103, 104, 128, 129, 196), and glucose uptake (1, 107, 117, 129, 131, 145). The intent of this study was to optimize existing electrical stimulation protocols to mimic the effect of short-term aerobic exercise training. In doing so, we would be able to study the effects of exercise in skeletal muscle cells of lean and severely obese individuals without the potential *in vivo* confounders inherent to the obese individual, such as exercise intolerance, endocrine, and humoral factors. Of the numerous electrical stimulation protocols available in the published literature, only a handful of studies have measured mitochondrial (28, 89, 107, 131, 188) or transcription factor (89, 188) protein responses to electrical stimulation that most closely resemble exercise training. Therefore these protocols were selected to establish our cell culture model of exercise training.

Human Subjects. Skeletal muscle was obtained by percutaneous biopsy from the vastus lateralis of lean (body mass index <25 kg/m^2) male subjects ages 18-30. Compared to lean individuals, obese subjects are more difficult to recruit and were therefore excluded from the initial development of the electrical stimulation model in HSkMC. 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. The experimental procedure and associated risks were explained, in detail, in written and oral format and informed consent was obtained. The study was approved by the East Carolina Policy and Review Committee on Human Research.

Primary Human Skeletal Muscle Cell Culture (HSkMC). The isolation and culturing of human primary skeletal muscle cells from biopsies was performed as previously described (15, 127).

Myoblasts were grown to ~80% confluence before deplating and freezing down in aliquots of ~500 k cells in growth media supplemented with 0.05% DMSO. Cell aliquots were allowed to slowly cool at -80°C in a "Mr. Frosty" (ThermoScientific, Nalgene) for 24 h before cryopreservation in liquid nitrogen. After thawing cell aliquots and sub-culturing to ~90% confluence, differentiation was induced by replacing growth media with low-serum differentiation media (DMEM, 2% heat-inactivated horse serum, 0.5 mg/ml BSA, 0.5 mg/ml fetuin, and 50 μg/ml gentamicin or penicillin/streptomycin). Media was changed every 48 h unless otherwise noted. All human myotube experiments were performed on day 7 or 8 of differentiation.

C2C12 Cell Culture. C2C12 cells were obtained from the American Type Culture Collection (ATCC) and cultured in growth media (high-glucose DMEM supplemented with 10% FBS, 0.5 mg/ml BSA, 0.4 mg/ml fetuin, 50 μg/ml penicillin/streptomycin) at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2 on an uncoated T-75 flask. After reaching ~80% confluence, myoblasts were trypsinized and sub-cultured onto 6-well plates containing a 1% gelatin coating at a density of 80 x 10^3 cells per well. After achieving ~90% confluence, differentiation was induced by replacing growth media with low-serum differentiation media (DMEM, 2% heat-inactivated horse serum, 0.5 mg/ml BSA, 0.5 mg/ml fetuin, and 50 μg/ml penicillin/streptomycin). Media was changed every 48 h. Experiments were initiated on day 5 of differentiation and harvested on day 9.

Chronic Stimulation of Myotube Cultures. Two types of electrical pulse generators and two types of stimulator lids were utilized in this investigation. Custom-made simulator lids and a bioreactor pulse generator were purchased from Kenneth Donnelly, PhD University of North

Carolina Chapel Hill (42). The stimulator lids were designed to fit a six-well culture dish and consisted of 12 steel electrodes per plate with two electrodes per well. Each electrode was placed approximately 20 mm apart and sat parallel to one another in the plate. The pulse generator purchased from Donnelly et al (42) was capable of being programmed to produce pulse trains of various frequencies and lengths interspersed with varying periods of rest. For that reason the bioreactor from Donnelly was programmed to mimic the electrical stimulation protocols of Burch et al (28) and Pinheiro et al (145) in addition to that of Donnelly et al (42). An amplifier was purchased to provide power (ROLLS Mosfet Power Amplifier 200 Watt/70Volt RA21006) and maximize throughput. The bioreactor and amplifier were capable of powering up to four stimulator plates at one time. The stimulator plates containing steel electrodes purchased from Donnelly were utilized only in our initial experiments presented in Figure 2.1. Thereafter the second set of stimulator plates utilized in this study, the C-Dish (ION Optix), containing carbon, rather than steel electrodes, were utilized in all subsequent experiments to more closely replicate other electrical stimulation protocols in the literature while also reducing the potential for toxic contaminants released from corrosion of the steel electrodes into the media (178). The second pulse generator utilized, the CS Stimulator (Harvard Apparatus), is not capable of producing pulse trains interspersed with periods of rest. However, the biphasic waves in the protocols of Irrcher et al (89), Uguccioni et al (188), and Nikolic et al (131) do not contain rest periods in-between pulse trains (i.e., the pulse is uniform and constant); therefore, the CS Stimulator was utilized to replicate these protocols. All stimulation protocols were verified for pulse width, frequency, and voltage using an oscilloscope (PicoScope). No difference in waveform was noted between the stimulation plates containing steel electrodes and carbon electrodes or between the carbon electrode plates.

The duration of the cell stimulation protocols can be further categorized as intermittent or continuous. For example, protocols consisting of 3 h of electrical stimulation followed by 21 h of quiescence and another 3 h of electrical stimulation are classified as intermittent. Other protocols are classified as continuous as there are no periods of quiescence and electrical stimulation runs continuously for 48 h.

For all intermittent stimulation experiments, electrical stimulation was initiated on day 4 (HSkMC) or day 5 (C2C12) of differentiation. Differentiation media was changed (2 ml) 1 h prior to the start of electrical stimulation and the plates were fitted with the stimulator lids. The stimulation bouts lasted 3 h unless otherwise noted. After each bout of stimulation, the stimulator lids were removed and the cells remained quiescent until the media was again changed 1 h prior to the next stimulation bout. All cell harvesting was performed 15-21 h after the last stimulation bout unless otherwise noted to distinguish between the acute effects of the last stimulation bout and the accumulated effects of the repeated bouts (89).

Lipid oxidation. On day 7 of differentiation, myotubes were incubated for 3 h 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 or 250 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 (127). Cells were washed twice with PBS, harvested in 200 µl of 0.05% SDS lysis buffer, and stored at -80°C for determination of protein concentration.

Respirometry experiments. Myotubes were washed 2x with PBS and lifted from six-well culture dishes with 0.05% trypsin EDTA. This reaction was neutralized by adding 10% FBS to the cell

suspension and centrifuged for 10 min at 1000 rpm at room temperature. The cell pellet was then resuspended in growth media, counted using a hemocytometer, centrifuged again for 10 min, and re-suspended in room temperature respiration buffer (130 mM sucrose, 60 mM potassium gluconate, 1 mM EGTA, 3 mM magnesium chloride, 10 mM potassium phosphate, 20 mM HEPES, 0.1% BSA; pH 7.4). The cells were then treated with 3 μg/10^6 cells/ml digitonin (i.e., a mild, cholesterol-specific detergent) for 5 min at 37° C on an orbital shaker. This procedure selectively permeabilizes the sarcolemmal membranes while keeping mitochondrial membranes, which lack significant levels of cholesterol, completely intact. Following permeabilization, the myotubes were washed by centrifugation at 1000 rpm for 5 min to remove endogenous substrates in cells. The cells were then resuspended in respiration buffer at a concentration of (3.0×10^6 cells per 2.2 ml) and loaded into a respiration chamber (Oroboros Oxygraph-2K, Oroboros Instruments Corp., Innsbruck, Austria). Once oxygen concentration flux stabilized, substrates were sequentially added in the following order GM – glutamate [10mM] malate [2mM]; ADP [4mM]; Cyto C – cytochrome C [10μM]; S – succinate [10mM]; FCCP - Carbonyl cyanide p trifluoro methoxyphenylhydrazone [2μM]. Side-by-side experiments for Control and Stimulation were performed on cells from each subject. The most stable portion of the oxygen concentration slope was determined for each condition and normalized to cell count as in previous cell culture respiration studies (30, 50, 143).

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 1 h, and centrifuged at 20,000 g for 30 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 fluoride (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). Membranes were incubated for 1 h 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.

Glucose and Lactate Determination. Starting on day 6 of differentiation, media samples were collected every 12 h and immediately frozen at –80° C for the subsequent determination of glucose and lactate. Glucose and lactate were determined by oxidation reaction (YSI model 2300 Stat Plus; Yellow Springs Instruments, Yellow Springs, OH).

Statistical Analyses. Data are presented as mean ± SEM. Statistical analyses were performed using a 1-way ANOVA with Student-Newman-Keuls methods for analysis of significance among groups. The level of significance was set at $P < 0.05$.

Results

Videos of Myotube Contraction. Videos demonstrating myotube contraction under electrical pacing are available in the supplementary materials. Contracting human skeletal muscle myotubes paced with electrical pulses of 50 V 10 Hz (4 x 0.3 ms pulses delivered in 400 ms

trains with 3.6 s recovery) are demonstrated in Videos 2.1 & 2.2. Contracting C2C12 myotubes paced with the identical electrical stimulation parameters can be observed in Videos 2.3 & 2.4.

The Effect of Electrical Stimulation on Fatty Acid Oxidation with Methods adapted from Donnelly et al. To assess whether contractile activity *in vitro* would increase fatty acid oxidation (FAO), HSkMC were electrically stimulated 3 h/d for 3 d with stimulation parameters modified from Donnelly et al. (42) (50 V, 10 Hz, provided as 4 x 0.3 ms pulses delivered in 400 ms trains with 3.6 s recovery). FAO was measured 21 h following the last bout of stimulation to discriminate between the acute and chronic effects of stimulation (89, 188). We observed an initial trend (P=0.12) for FAO to increase in response to repeated electrical stimulation for 3 h/d for 3 d (Figure 2.1A). When we repeated these experiments the trend diminished (Figure 1B-C), however, when the outlier was removed from the data set, the trend reemerged (P=0.10) (Figure 2.1D). A larger sample size is necessary to determine whether this protocol is effective in increasing FAO in HSkMC. Additional experiments to determine whether this protocol was capable of effectively increasing FAO are not reported here as the technique utilized in our laboratory to measure FAO began producing inconsistent and unreliable results. The scintillation counts used to determine FAO decreased by a magnitude of four to five fold. Therefore, we felt the results produced by this technique were unreliable and the assay had lost its sensitivity to detect meaningful change. Despite numerous attempts to troubleshoot this technique over the course of the next six months, we were unable to re-establish this technique. To that end we were forced to move on to a different direction. The work of Uguccioni & Hood (188) provided an alternative protocol to address the aims of our project.

The Effect of Electrical Stimulation on Mitochondrial Content and Protein Synthesis with Methods adapted from Uguccioni & Hood. In addition to increasing the rate of FAO in skeletal muscle, exercise training is also known to increase mitochondrial content. In developing a cell culture model of short-term aerobic exercise training, we sought to utilize a protocol known to stimulate FAO and/or mitochondrial content. The electrical stimulation protocol of Uguccioni & Hood (3 h/d, 5 Hz, 9V, 4 d) has been shown to increase COX IV protein and the mitochondrial transcription factor A (TFAM) by 3.9 and 2.3 fold in C2C12 cells (188). When this protocol was applied to HSkMC, we observed no increase in COX IV protein (Figure 2.2A). Although we expected COX IV protein to increase in response to this protocol, a single protein does not provide an all encompassing picture of total mitochondrial content. Therefore, to assess a broader range of proteins we measured mitochondrial content using the OXPHOS cocktail (MitoSciences, Eugene, OR), which contains an antibody for a subunit of each complex of the respiratory chain. Here, we observed no difference in subunits from Complexes II, III, and V, and actually observed a significant decrease in protein from the subunits of Complexes I and IV ($P<0.05$) in the electrically stimulated cells (Figure 2.2B).

The pulse generator, stimulation lids, and cell type utilized by Uguccioni and Hood (89, 188) were different from those used in our study. To assess whether the electrical stimulation set-up or cell type may have affected the ability to replicate the findings of Uguccioni and Hood (188), we electrically stimulated C2C12 myotubes 3 h/d for 4 d and again found no increase in COX IV protein (Figure 2.3A). There was also no increase in Complex I, II, III, or V proteins with electrical stimulation (Figure 2.3B), indicating the protocol did not have a significant effect on mitochondrial content.

Aerobic training and strength training are traditionally thought to elicit divergent responses, i.e. increased oxidative capacity or increased synthesis of myofibril proteins. In isolated rat muscle, Atherton et al (6) demonstrated that altering the frequency of stimulation, 10 Hz versus 100 Hz, resulted in aerobic and resistance training like responses, respectively. To assess whether the conditions of our experimental set-up and stimulation parameters were activating the protein synthesis pathways associated with resistance training, we measured several proteins involved in the regulation of protein synthesis (AKT-1, eEF2, mTOR, p70-S6, and 4E-BP1). Electrical stimulation did not increase these proteins (Figure 2.3C).

The Interaction of Electrical Stimulation and Antibiotic on Mitochondrial Protein in Skeletal Muscle Cells. The use of antibiotics in cell culture media is a standard practice to prevent bacterial and/or fungal contamination. Two of the most common antibiotics in tissue culture, *gentamicin* and *streptomycin*, are in a class of antibiotics known as aminoglycosides. Although aminoglycosides are highly effective in preventing bacterial contamination, a major limitation of their use in tissue culture is altered calcium kinetics and decreased contractility (44, 76, 114). On the other hand, the antifungal agents commonly used in tissue culture, Amphotericin B and penicillin, seem to have little effect on muscle contraction (131). Although both gentamicin and streptomycin decrease contractility, streptomycin is thought to be less potent in inhibiting contraction (12), therefore in our next set of experiments we sought to investigate whether replacing gentamicin with streptomycin would augment the response to our contraction stimulus. To that end we cultured C2C12 myotubes in either a) gentamicin/Amphotericin B or b) penicillin/streptomycin and electrically stimulated them according to the protocols of Uguccioni & Hood (188), Burch et al (28), Donnelly et al (42), and Pinheiro et al (145). Neither, the type of antibiotic, or the electrical stimulation protocol had any effect on COX IV protein using the

protocols of Uguccioni & Hood (188), Burch et al (28), or Donnelly et al (42) (Figures 2.4A, B, and C) whereas COX IV protein significantly increased ($P < 0.05$) with the protocol of Pinheiro et al in the presence of penicillin/streptomycin but not gentamicin (Figure 2.4D). These data suggest that, during these conditions, gentamicin was prohibiting some of the mitochondrial adaptations we were attempting to initiate. Follow up experiments using the protocol of Pinheiro et al in HSkMC failed to increase proteins from Complexes I, III, or V as well as COX IV protein despite the absence of gentamicin from the culture media (Figure 2.5).

The Effect of Continuous 48 h Electrical Stimulation on Glucose and Lactate Content in the Media, Mitochondrial Protein Content, and Oxygen Consumption. Initially, we only utilized intermittent electrical stimulation protocols because they most closely resemble the physiological demands of exercise *in vivo* (28, 42, 145, 188), that is, exercise bouts interspersed with periods of rest. However, these protocols were ineffective in eliciting training-like adaptations. Continuous, uninterrupted (24-48 h), electrical stimulation offers an alternative to intermittent contractile bouts and has been shown to increase some indices of mitochondrial content (28, 107, 131). Several investigators have also demonstrated that continuous stimulation enhances myotube maturation (56, 107, 129) by optimizing sarcomere alignment. We hypothesized that by electrically stimulating HSkMC continuously over the course of 48 h, components of the excitation contraction machinery would become more mature/aligned, thereby increasing the number of cells recruited during the course of stimulation and increasing energy demand. To assess whether electrical stimulation was increasing energy demand and utilization, we measured glucose and lactate in the cell media at baseline and at 12 h intervals up to 48 h. As expected we observed a significant increase in lactate accumulation in the stimulated cell media at all time points post baseline ($P < 0.05$) (Figure 2.6A). However, we did not observe a significant effect

of time, potentially indicating our continuous stimulation was not further inducing sarcomeric development and muscle cell recruitment differentiation. Interestingly we observed no difference in media glucose concentration between groups at any time point (Figure 2.6B). To determine whether our intervention affected the oxidative capacity of the myotubes we measured OXPHOS protein content and respiration immediately after the 48 h stimulation bout. There were no differences between control and stimulated cells in either OXPHOS protein content (Figure 2.6C) or oxidative capacity (Figure 2.6D).

The Effect of Lipid and 48 h Continuous Electrical Stimulation on FAO in HSkMC. Skeletal muscle increases the rate of FAO during aerobic exercise (192). The increased demand for fatty acids during exercise training is met from the mobilization of fatty acids from adipose stores and the breakdown of intramuscular triglyceride stored within the skeletal muscle. In the culture model there is a very limited amount of lipid available in the media (68). To more accurately resemble the energetic environment of skeletal muscle during rest and exercise, 100 μM oleate/palmitate (O/P) was added to the cell media for 48 h, with (Stim w/Lipid) and without 48 h of continuous electrical stimulation (Lipid). In the present investigation our initial question was whether electrical stimulation was capable of mimicking short-term exercise training by increasing FAO in HSkMC. The radiolabeled FAO procedure is the most effective tool in answering this question but was abandoned after the technique became unreliable (Figures 2.1A-D). Here we reinitiated our troubleshooting attempts and were successful in re-establishing the radiolabeled FAO technique. Lipid, electrical stimulation (Stim), and Stim w/Lipid significantly increased FAO ($P < 0.05$) above control values (Figure 2.7A-B). FAO was greater in Lipid and Stim w/Lipid ($P < 0.05$) compared to stimulation alone. There was no difference in FAO between Lipid and Stim w/ Lipid, suggesting the presence of lipid during electrical stimulation

did not further enhance FAO; the increase in FAO with electrical stimulation was similar under control (16%) and lipid (22%) conditions (Figure 2.7B & C).

Discussion

The purpose of this investigation was to create an electrical stimulation model in cultured human skeletal muscle cells (HSkMC) capable of replicating the metabolic adaptations to short-term exercise training observed *in vivo.* Developing such a model would be advantageous in understanding the molecular responses to exercise training without the conflicting effects of neuro-endocrine and exercise intolerance concerns observed in the obese population.

Although the results of the current investigation do not establish a model of exercise training in culture, a closer examination of the data in this investigation and previously published literature reveals several key observations: **1)** the lack of standardized culture conditions and electrical stimulation parameters contribute to inconsistent electrical stimulation outcomes, **2)** the evidence for short-term *in vitro* intermittent (3 h/d, 3-4 d) electrical stimulation to enhance oxidative enzyme content or activity is scarce and difficult to reproduce (89, 188), **3)** continuous electrical stimulation of 48 h is capable of increasing FAO, and **4)** periods of continuous stimulation in skeletal muscle myotubes greater than 48 h may be required to enhance mitochondrial content and oxidative capacity characteristic of *in vivo* short-term aerobic exercise training.

Methodological Considerations for Electrical Stimulation of Skeletal Muscle Cells in Culture. There is a vast array of electrical stimulation protocols in skeletal muscle cells in the published literature. The combination of inherent differences in skeletal muscle characteristics among cell types including excitability, capacity to differentiate, and an infinite number of electrical

stimulation permutations and variables contribute to inconsistent electrical stimulation outcomes. For example, a number of key parameters vary considerably among electrical stimulation investigations in the published literature: the frequency of stimulation ranges from 0.25 to 100 Hz, voltage from 4 to 65 V, pulse width from 0.15 to 24.00 ms, and hours of stimulation from 1.5 to 48 hrs (Table 2.1). Other important considerations include but are not limited to the number of stimulation days, the day of differentiation to begin stimulation, the length of the electrical pulse train and rest period, the amount of electrical current, material of the plate matrix, antibiotic selection, serum type and concentration of the media, electrode material, distance between electrodes, time of measurement, and the type of pulse generator and plates. It is understandable that diverse research questions require distinct electrical stimulation parameters, however, the sheer number of variables involved in the electrical stimulation of cultured cells highlights the difficulties in reproducing meaningful results between and within laboratories.

There are a number of custom made (1, 35, 103, 104, 131, 188) and commercially available electrical stimulation pulse generators utilized in electrical stimulation studies with the most common commercially available stimulators being the C-Pace EP (IonOptix, Milton, MA) and Grass Stimulators (Grass Technologies, Warwick, RI). The type of stimulator should not make an appreciable difference in electrical stimulation results as voltage is typically verified with an oscilloscope. The type of electrode utilized in the experiments will, however, affect how efficiently the current is transferred through the cell media. There are several commonly used electrodes composed of various materials including copper, brass, titanium, stainless steel, platinum, and carbon (117, 178) each possessing different charge transfer characteristics. This is important to note because although voltage is reported in the literature, current typically is not. Voltage is a measure of the difference in electrical potential between two electrodes but provides

no indication of how efficiently the charge is transferred to the media or the cultured cells (29, 177, 178). Manabe et al (117) found that copper, brass, and platinum electrodes did not cause an efficient contraction in C2C12 cells, although carbon electrodes did. In contrast, numerous investigators have found platinum electrodes to be effective in inducing metabolic responses, including Ca^{2+} transients (103, 190) and glucose uptake (1, 145, 190), measures that Manabe et al (117) reported. With that being said, the field seems to be promoting carbon electrodes as the gold standard due to their superior charge transfer characteristics and lack of electrode corrosion (177, 178). In addition, several recent investigations utilizing carbon electrodes have been successful in electrically stimulating human primary skeletal muscle cells (107, 131, 180), therefore the use of carbon electrodes is warranted in future electrical stimulation investigations.

The type of antibiotics in the cell culture media may also play a role in the response to electrical stimulation. The use of antibiotics is a standard practice to prevent bacterial and/or fungal contamination. The two most commonly used antibiotic combinations in tissue culture are a) *gentamicin* and another agent such as Amphotericin B or b) a combination of *penicillin/streptomycin (P/S).* Amphotericin B and penicillin function as antifungal agents whereas gentamicin and streptomycin are classified as aminoglycosides whose purpose is to prevent bacterial contamination. Although aminoglycosides are highly effective in preventing bacterial contamination, a major limitation of their use in muscle tissue is altered calcium kinetics and decreased contractility (44, 76, 114). Although both gentamicin and streptomycin decrease contractility, streptomycin is thought to be less potent in inhibiting contraction (12). To determine whether antibiotics were affecting our electrical stimulation results we utilized a combination of (P/S) in a number of intermittent stimulation trials (Figure 2.4A-D) and found that in the presence of P/S, the Pinheiro et al (145) electrical stimulation protocol significantly

increased COX IV whereas the presence of *gentamicin* completely abolished this effect (Figure 4C). When the Pinheiro protocol was repeated in HSkMC, there was no increase in COX IV protein, underscoring the importance of differences between cell types. In the literature there are reports indicating positive results in electrical stimulation studies with (117, 131, 188) and without (107, 180) the presence of *penicillin/streptomycin*. However, there are no published reports in which *gentamicin* was the antibiotic utilized, therefore, one of the main findings of this work was the indication that it may be fruitful to utilize *penicillin/streptomycin* or no antibiotic when utilizing electrical stimulation in cell culture systems.

The inherent properties of the type of myocyte utilized may also have a large impact on whether the electrical stimulation protocol induces a desired response. Burch et al (28) reported that SOL8 cells, an immortalized cell line from mouse soleus, did not increase PGC-1α mRNA in response to their electrical stimulation protocol (24 h, 50 Hz 14V, 1 sec pulse 1 sec rest) and that mouse primary cells did not survive their protocol whereas C2C12 cells demonstrated a robust PGC-1α mRNA response. Manabe et al (117) also found that C2C12 cells responded to their stimulation protocol by efficiently contracting whereas L6 cells did not.

Differences in the degree of excitability between cultured cell lines may have prevented any appreciable change in the desired metabolic outcomes in the present investigation. It is well documented that the excitability of cultured cells is greatest in rat and mouse primary cells and that C2C12 cells possess "exceptionally low excitability" (41). The excitability of HSkMC is several orders of magnitude less excitable than C2C12 cells (Supplementary Videos). Manabe et al (117) estimated that approximately 80% of C2C12 myotubes contracted upon electrical stimulation. In the present investigation, we estimate C2C12 contraction to be 50-75% of

myotubes, while HSkMC contraction was rarely ≥ 10% with no visible contraction noted on some occasions.

The degree of muscle tissue excitability is regarded as an effective marker of muscle maturation (41). The low level of excitability in HSkMC is most likely due to a poor degree of maturation and is a reflection of its primitive state. Human primary skeletal muscle cells are not simply adult muscle cells in a culture plate. There are a number of transitional stages of development immature skeletal muscle cells must undergo to reach the adult state. For example, the development of skeletal muscle *in vivo* is characterized by the sequential expression of embryonic, neonatal, and adult myosin heavy chain (MHC) forms (9). In culture, human primary myotubes are in their infancy. For example, comparative gene expression profiling between human primary myotubes and adult skeletal muscle shows that genes associated with mitochondrial pathways of energy production, myofilaments, and calcium handling are all downregulated in cultured cells (150). Dennis & Dow (41) found that the degree of skeletal muscle tissue excitability in rodents was proportional to the degree of muscle maturation. Excitability increased at each step of the development process starting with rat primary skeletal muscle cells, followed by neonatal muscle of 10-14 d, 25 d, and 40-44 d, and greatest in adult skeletal muscle (41). Compared to skeletal muscle cells from chicken, rat, and mouse, human primary skeletal muscle cells are thought to differentiate less rapidly and less completely than their animal counterparts (40). Furthermore, based upon myotube excitability, it appears that rat and mouse primary cells, as well as those of immortalized cell lines may be capable of reaching a more terminal degree of differentiation in culture than HSkMC.

Another factor that may be affecting the results of studies in HSkMC is myotube heterogeneity. Compared to immortalized cell lines, the differentiation of primary cells is

considered to be heterogeneous (18, 115), which is thought to contribute to a considerable degree of variability in experimental results (18, 115, 117). In the present study, only the most highly differentiated myotubes would visually contract upon electrical stimulation. When variations of numerous electrical stimulation protocols were applied (> 25 variations) the myotubes capable of contracting would do so regardless of the stimulation parameters, provided the voltage was greater than a minimum threshold of 1.5 V (unpublished observations). These observations, along with the vast number of published electrical stimulation protocols, suggest that an optimal stimulation protocol has not yet been achieved, and furthermore may not be necessary to develop.

It may be beneficial to identify ways to increase HSkMC differentiation to increase the number of myotubes capable of contracting with sufficient stimuli. There are a number of options for increasing the degree of myotube differentiation in cultured skeletal muscle cells including replacing the horse serum in the differentiation media with fetal calf serum (66) or the serum substitute Ultroser G (13, 65, 69, 147), adding insulin like growth factor-1 (IGF-1) (91) or a cholinergic agonist (102) to the differentiation media, or changing the culture plate matrix from collagen to extracellular matrix gel (ECM-gel) (65) or Matrigel (46, 108). Each of these factors has been shown to greatly enhance skeletal muscle differentiation, however it is unknown whether the enhanced differentiation translates into a more robust contraction. Collectively, it appears as though efforts should be made to enhance myotube differentiation to increase the effectiveness of the electrical stimulation protocol.

The removal of serum from the media during the electrical stimulation period may also aid in obtaining a more terminally differentiated culture by reducing the percentage of myoblasts. For example, Lambernd et al (107) significantly increased OXPHOS protein after just 24 h of

stimulation in HSkMC after serum starving the night before and during the 24 h of stimulation. In normal culture conditions, the removal or reduction of serum from the media inhibits myoblast proliferation and induces myogenesis (98). Although a reduction in serum promotes myoblast differentiation, a subset of cells are resistant to differentiation and remain myoblasts (18, 209). In addition, electrical stimulation of myocyte cultures (26, 135) and stretching (194) are known to enhance myoblast proliferation. It is possible that any measureable effects of electrical stimulation on myotube cultures are, in effect, "drowned out" by the proliferating myoblasts. Taken together, serum starvation provides a favorable environment for electrical stimulation to enhance myotube maturation while at the same time limiting myoblast proliferation.

Mitochondrial Responses to Electrical Stimulation in Culture - Intermittent Electrical Stimulation. Although there are a number of electrical stimulation investigations in cell culture, only a handful of these studies have assessed mitochondrial protein or activity (28, 89, 90, 107, 131, 188), and of this subset, only the Hood laboratory (89, 188) has reported increased mitochondrial content (in C2C12 cells) in response to intermittent stimulation protocols of 3 h/d for 2-4 d. In the current investigation, we were unable to replicate the findings of Uguccioni & Hood (188) (5 Hz, 9 V) in HSkMC or C2C12 myotubes. In the current investigation, 5 Hz, 9 V may have actually decreased the oxidative machinery in HSkMC as indicated by decreased Complex I and IV subunit protein (Figure 2.2B). Other investigators have reported that high voltage or frequencies above 3 Hz will result in tetanus (personal observations, 17) and kill C2C12 muscle cells (42). In another of Hood and colleagues' electrical stimulation studies, they (89) reported utilizing 5 Hz and 55 V to promote mitochondrial biogenesis. When 5 Hz and 30 V were applied to HSkMC in the current investigation mitochondrial content was not measured

because the myotubes were badly damaged after 1 d of stimulation and completely destroyed after 2 d (data could not be collected). The published accounts of intermittent electrical stimulation capable of increasing oxidative capacity (89, 188) were either not reproducible (present investigation) or resulted in cell death (17).

In addition to the Hood studies, three alternative intermittent protocols utilized in this investigation failed to increase mitochondrial content (28, 42, 145). The Donnelly et al. (42) protocol was originally utilized as a single, 3 h acute bout in C2C12 cells to demonstrate the protocol's effectiveness in studying metabolic responses to contractile activity. The efficacy of their protocol was verified by demonstrating an increase in CAMKII and AMPK phosphorylation as well as an increase in PGC-1α mRNA (142), all of which are classic responses to *in vivo* contractile activity (8). These same investigators later observed a similar increase in PGC-1α mRNA in electrically stimulated human primary myotubes (data not shown), demonstrating that, like immortalized cell lines, cultured human skeletal muscle cells are also metabolically responsive to this stimulation protocol. PGC-1α is a powerful regulator of oxidative phosphorylation and β-oxidation genes, therefore we hypothesized that by expanding the original protocol to encompass three bouts of electrical stimulation rather than one, we would observe an increase in FAO and/or mitochondrial content in human primary myotubes. In addition to Donnelly et al (42), Burch et al (28) previously demonstrated a transient increase in PGC-1α mRNA in response to electrical stimulation, however in the current investigation neither protocol translated into increased mitochondrial protein content (Figures 2.4B & D). The duration of electrical stimulation (3 h/d, 3 d) may have been insufficient to increase mitochondrial content. When the Burch et al protocol (28) was applied continuously for 24 h in C2C12 cells, PGC-1α, cytochrome c, and medium chain acyl-CoA dehydrogenase protein

increased. It is remains unclear whether short-term intermittent stimulation is a feasible model for studying mitochondrial biogenesis in HSkMC.

Mitochondrial Responses to Electrical Stimulation in Culture - Continuous Electrical Stimulation. Initially, only intermittent electrical stimulation protocols (28, 42, 145, 188) were utilized in the current investigation as they more closely resemble the physiological demands of exercise *in vivo*, however, these protocols were ineffective in eliciting mitochondrial adaptations. Although not as physiologically relevant, continuous, uninterrupted (24-48 h), electrical stimulation has been shown to increase some indices of mitochondrial content (28, 107, 131), enhance sarcomeric development and alignment (56, 107), and in some cases facilitate a transition to a more oxidative fiber type (28, 45, 131, 196). In the current investigation, 48 h of electrical stimulation failed to increase OXPHOS protein (Figure 2.6C). In contrast to our data, Nikolic et al (131) reported increased mitochondrial content as visualized with MitoTracker after 48 h of stimulation in HSkMC. However, these data must be interpreted with caution, as the same investigators failed to report changes in citrate synthase activity (CS) or the OXPHOS proteins. The duration of the Nikolic et al (131) intervention and that of the present investigation may not have been of a sufficient duration to increase mitochondrial content. In a 7-10 d training study in humans, Battaglia et al (10) observed no increase in OXPHOS protein or β-HAD activity, whereas CS activity did increase in skeletal muscle tissue. On the other hand, several investigators have noted no increase in several indices of mitochondrial content (SDH, CS, β-HAD) after short-term exercise training for 3 d (70), 5 d (141), 5-7 d (72), or 10-12 d (74) whereas longer training durations of 14 d (176) and 31 d (141) robustly increase mitochondrial content in skeletal muscle tissue. In the culture system, long-term electrical stimulation may be required to produce oxidative changes; however, this may not be feasible as HSkMC are thought

to possess a finite (3-4 d) experimental lifespan (66). On the other hand, skeletal muscle cells cultured from embryonic chick breast muscle (45) and the hindlimb musculature of newborn rats (128) have been electrically stimulated in culture for up to 19 and 20 days respectively. Human skeletal muscle myotubes are capable of being maintained in culture for up to three weeks (13, 170) and, with such an extended culture duration, more closely resemble adult muscle in terms of enzymatic activity and myosin heavy chain than do their 7 d differentiated counterparts. However it is unclear whether these cells are capable of withstanding the forces imposed by electrical stimulation without lifting from the culture plate. After 24 and 48 h of electrical stimulation there is a noticeable amount of cell lifting off the culture plate, however there are no appreciable differences in total protein content between control and stimulated cultures (data not shown). A potential area for future investigations to pursue in developing a short-term training contractile activity model in HSkMC may be to extend the culture beyond 7 or 8 days of differentiation and subsequently electrically stimulate the culture for > 48 hrs.

In the present investigation, 48 h of electrical stimulation failed to increase OXPHOS protein (Figure 2.6C) or respiration in permeabilized myocytes (Figure 2.6D), it did however, increase the concentration of lactate in the media at each time point (Figure 2.6A). Although media glucose concentration in the present study (Figure 2.6B) did not differ between the control and stimulated groups, the ability of electrical stimulation to increase glycolytic metabolism (145) and glucose oxidation appears to be more robust than its ability to increase FAO (107, 131, 180). Nikolic et al (131) observed a ~50% increase in glucose oxidation and ~10% increase in FAO when measured during the two hours immediately following 24 h of electrical stimulation. In this same investigation, glucose uptake was significantly increased at 24 and 48 h in response to electrical stimulation whereas oleate uptake remained unchanged. Furthermore, it was not

until after 48 h of electrical stimulation that FAO significantly increased, however, the increase remained far below that of glucose (90% vs. 35% increase), indicating a greater reliance on glucose oxidation. In a similar investigation in HSkMC, short-term electrical stimulation increased glucose oxidation whereas 24 h of continuous stimulation had no effect on either palmitate or oleate oxidation (107). Short-term aerobic exercise training (7-10 d) *in vivo* decreases the reliance on CHO and increases the reliance on fatty acids (10, 14). During the transition to a greater reliance on fatty acids there is generally also a decrease in muscle lactate accumulation. In the present investigation, media lactate significantly increased at 12 h and remained elevated throughout the 48 h stimulation period suggesting the duration of stimulation was insufficient to induce a substrate switch characteristic of *in vivo* short-term training.

In culture, human skeletal muscle cells possess fewer mitochondria and derive a greater proportion of their energy needs from glycolysis compared to skeletal muscle *in vivo* (150, 158, 189). The addition of lipid to cultured cells increases FAO in HSkMC derived from lean individuals (64, 99). Based on preliminary experiments, we hypothesized that the inclusion of lipid may be necessary to promote the use of oxidative metabolism during electrical stimulation. In this set of experiments, myotubes were incubated in 100 μM oleate/palmitate for 48 h with or without 48 h of continuous electrical stimulation (Figure 2.7A). A number of observations from these experiments can be made 1) electrical stimulation alone can increase FAO and 2) the presence of lipid may not facilitate an increase in FAO as electrical stimulation with and without the presence of lipid resulted in a similar (~20%) increase in FAO.

The ability for continuous electrical stimulation alone to increase FAO is interesting and seemingly conflicts with the permeabilized myotube data collected earlier in this investigation in which 48 h of continuous stimulation did not increase OXPHOS protein or respiratory capacity

(Figures 2.6C & D). At first glance, these results seem completely at odds, however they may be reconciled through differences in methodology. In the initial continuous electrical stimulation data set (Figures 2.6A-D), respiratory capacity was measured utilizing the permeabilized cell technique. In this technique, the cell membrane is permeabilized, all endogenous substrate is removed, and oxidizable substrates are sequentially titrated in to test the "capacity" of the oxidative phosphorylation system: endogenous substrates and allosteric regulation have no effect on substrate selection. On the other hand, our data set indicating that electrical stimulation increased FAO (Figure 2.7A) was collected in adhered, intact myotubes where endogenous substrates are not removed and intracellular regulation may have a profound effect on substrate selection.

In the permeabilized cell data set (Figures 2.6A-D), lactate concentration in the cell media was significantly elevated at each time point (every 12 h), indicating an increased reliance on glycolytic, rather than oxidative metabolism. In this data set, glucose concentration in the media was not different between the stimulated and the control cells; therefore it is plausible the increased lactate concentration was the result of increased glycogenolysis rather than glucose uptake. An increase in media lactate concentration was also observed in the intact data set (data not shown) suggesting glycogenolysis could have potentially lead to a large enough reduction in glycogen to promote the increase in FAO observed in the intact cells (Figures 2.7A & B).

Alternatively, an increase in FAO may not be due to decreased glycogen stores, and is instead a very early adaptation to short-term aerobic training. In the present investigation, FAO was measured approximately 15 hours after the termination of the 48 h stimulation bout. Although glycogen content was not measured, it is plausible that glycogen content was replenished during this time. An early response to short-term aerobic training *in vivo* is an

increase in the monocarboxylate transporters (MCT) (70, 71), which are responsible for exchanging pyruvate for lactate across the sarcolemma. In the present investigation, elevated media lactate may have been the result of increased MCT expression and exchange of media pyruvate for myocyte lactate, rather than increased glycogenolysis. Although MCT expression and increased FAO are likely not directly related, both are markers of an early response to short-term aerobic training. Together this would suggest that 48 h of stimulation enhances FAO before changes in oxidative capacity appear and occur in a fashion similar to short-term exercise training, however, the precise mechanisms underlying the increase in FAO are currently unknown.

The inability for the presence of lipid to further increase FAO from electrical stimulation compared to lipid alone may be reflective of skeletal muscle's preference for CHO (16) and is in agreement with previous findings. Taube et al (180) incubated HSkMC from lean individuals in 100 μM palmitate or 100 μM palmitate/adipocyte conditioned media for 24 h, and found that when electrical stimulation was simultaneously applied for twenty-four hours intracellular triglyceride and FAO remained unchanged, indicating electrical stimulation was unable to rescue FAO *in vitro*. Data from the present and previous investigations (107, 131, 180) suggest that contractile activity initially increases the reliance on CHO and increased FAO may be a secondary benefit if the duration of electrical stimulation is of a sufficient length of time (≥ 48 h). For the full effects of short-term aerobic training and increased skeletal muscle oxidative capacity to be observed, future electrical stimulation investigations in HSkMC should extend the stimulation period beyond 48 h.

Limitations. There are several limitations in the present investigation and inherent to the *in vitro* model of contractile activity in human primary skeletal muscle cells: 1) The small sample sizes

utilized in the present investigation make conclusions from this study difficult to draw and comparisons to other investigations difficult to make; 2) HSkMC are extremely unexcitable, C2C12 cells are characterized by having an exceptionally low level of excitability and the excitability of HSkMC is several orders of magnitude lower than that of C2C12 cells; and 3) HSkMC are characterized by a heterogeneous degree of differentiation between and within subjects (18, 115), which may contribute to inconsistent results (117). Myoblasts (126) and myotubes are capable of appropriately responding to lipid incubations by upregulating FAO regardless of the degree of differentiation or lack thereof. On the other hand, myotubes must be fully differentiated to respond to electrical stimulation, and even then, they may be unresponsive. For skeletal muscle myocytes to consistently contract all aspects of excitation contraction coupling must be intact including the ability to initiate and propagate an action potential, release and sequester calcium, and properly develop and align sarcomeric proteins, aspects of which tend to be downregulated in HSkMC (150). Only the most terminally differentiated myotubes are capable of contracting, and oftentimes only represent ~10% of the total culture. A major drawback to myotubes with the degree of differentiation necessary to contract is they tend to spontaneously contract without external stimulation (66), resulting in a loss of experimental control when both the control group and the stimulated group are contracting. In this situation, inhibiting spontaneous myotube contraction in the control group with tetrodotoxin or another paralyzing agent is not an option as this has been shown to alter metabolic enzyme activities (90, 109, 130).

Electrical stimulation has been shown to enhance sarcomeric development and contraction (56, 107), however a fine line must be navigated to obtain the desired metabolic outcome. For example, if myotubes are not differentiated to a great enough degree they will not

contract and the metabolic response will be blunted. If myotubes are too differentiated the control myotubes will spontaneously contract and both control and stimulated culture plates will lose myotubes from cell detachment (69, 147). Although electrical stimulation has been shown to improve sarcomeric development, initiating electrical stimulation too early will result in a reduction in myotube striation (112) and a loss of function. In addition, the currently accepted experimental window within which HSkMC are viable is limited to 4-5 d between days 4 and 8 of differentiation, insufficient time to reach complete maturation. In rat primary culture, Naumann & Pette (128) showed that long term differentiation greater than 10 d was necessary for the myotube cultures to express adult form myosin heavy chain and even after 37 d of culture adult myosin heavy chain represented ~50**%** of total myosin indicating that a fully differentiated adult state is not reached *in vitro*. However, despite the aforementioned functional limitations of investigating the metabolic effects in HSkMC, several investigators (107, 131, 180) have been able to demonstrate that *in vitro* electrical stimulation is a viable model for the investigation of skeletal muscle contractile activity.

Summary. Myotube excitability determines whether a skeletal muscle cell is capable of contracting in response to electrical stimulation, which, in turn, is determined by the degree of myotube differentiation. There are an infinite number of electrical stimulation protocols in the scientific literature and the vast majority of these protocols are capable of eliciting contractile activity, but only in the most differentiated myotubes in culture. Therefore, the focus of future investigations on the effects of electrical stimulation on HSkMC metabolism should target factors enhancing myotube maturation (Ultroser G, IGF-1, ECM-gel) to ensure robust responses from the cultured cells. The use of carbon electrodes is warranted due to their superior charge transfer characteristics and biocompatibility. The presence of antibiotic may have a deleterious

effect on contractile activity so we would therefore recommend it be excluded from the cell media in future electrical stimulation investigations in HSkMC. In addition, the concentration of serum combined with the effects of electrical stimulation promote myoblast proliferation, possibly diluting the desired metabolic response and therefore serum should also be eliminated or at the very least future experiments should delineate the effects of reductions in serum during electrical stimulation.

In regards to the stimulation protocol, the results of the present investigation and previous literature (107, 131, 180) indicate that continuous electrical stimulation (24-48 h) may be superior to intermittent simulation in its ability to increase mitochondrial content and alter substrate metabolism in HSkMC. However, the metabolic outcomes associated with 48 h of continuous electrical stimulation in the current investigation were not able to fully replicate many of the short-term training adaptations. Continuous electrical stimulation significantly increased lactate concentration at 12 h and remained elevated for the entire stimulation period (48 h). The failure to reduce media lactate concentration, increase OXPHOS protein, or oxidative capacity is an indication that our protocol has not yet induced measurable training effects at the level of mitochondrial content. The increase in FAO observed ~15 h after the 48 h stimulation period may be a signal of a very early short-term training adaptation. However, from the results contained herein, the HSkMC contractile activity model may be more suited to explore the acute effects of exercise (single 3 h bout) rather than short-term training effects. For the short-term training effects to be explored future investigations should look for avenues to enhance myotube differentiation and thereby increase the number of myocytes recruited and capable of contracting or to increase the training duration from 48 h to $\geq$ 72 h. Forty-eight hours

of continuous electrical stimulation most closely resembles a single prolonged exercise bout, for the effects of short-term training to be observed, the duration of the protocol must be extended.

Grants

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

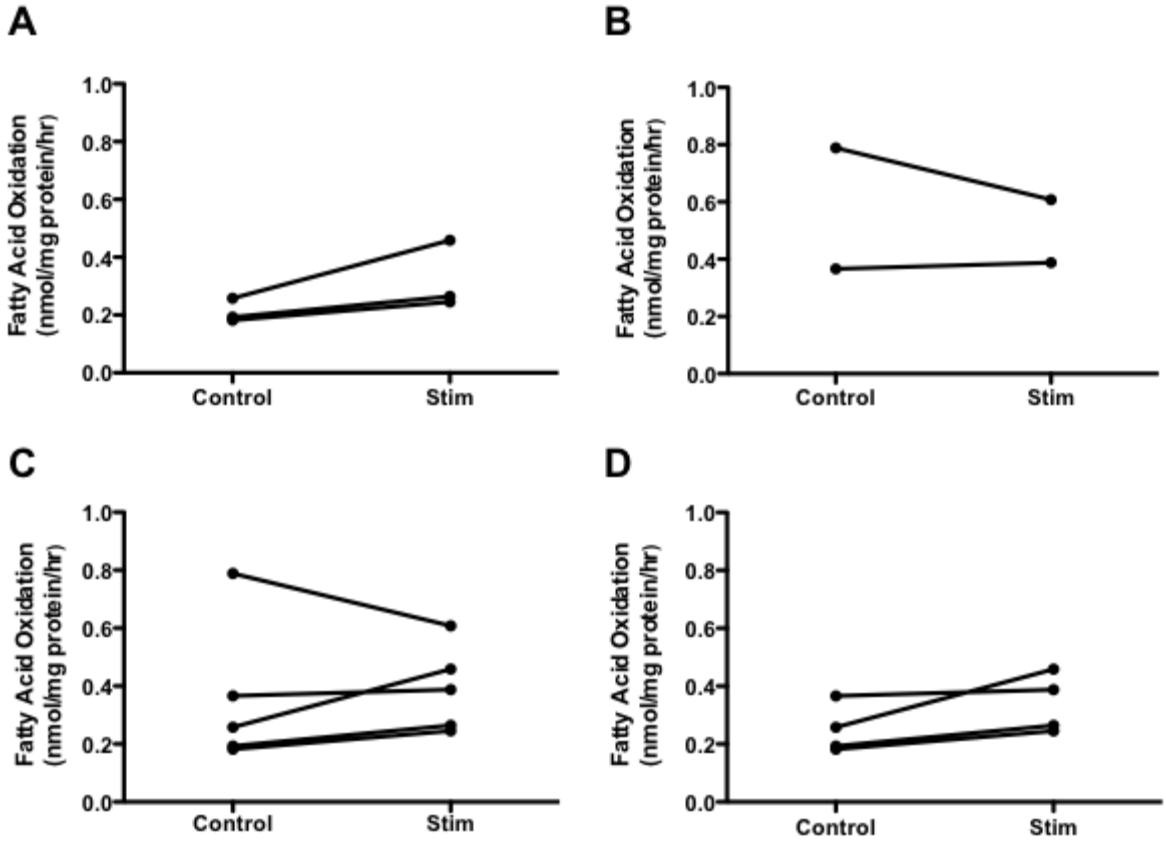
Disclosures

The authors have nothing to declare.

Table 2.1. Range and Reported Values of Selected Electrical Stimulation Parameters from Scientific Literature in Cultured Cells

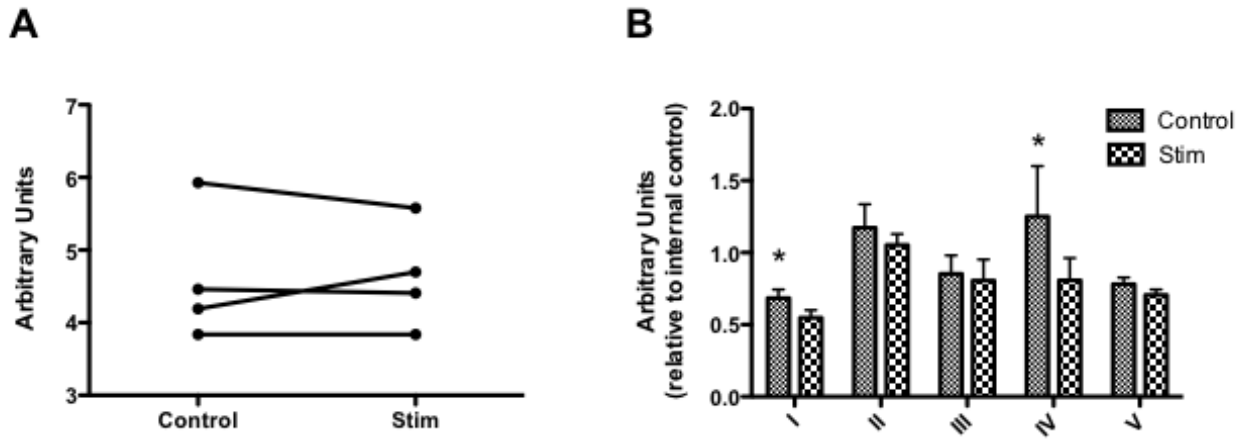
	Range of Selected Electrical Stimulation Parameters in Cultured Skeletal Muscle													
Hertz (Hz)	0.25	0.5	0.94	1	2	2.5	3	5	6.25	10	25	45	50	100
Voltage (V)	4	9	10	11.5	14	16	18	20	30	40	50	55	65	
Pulse Width (ms)	0.15	0.2	0.4	1	2	2.5	3	4	10	24				
Time (hrs)	1.5	2	3	4	8	12	24	48						

Figure 2.1. The effect of intermittent electrical stimulation on fatty acid oxidation in human primary myotubes.



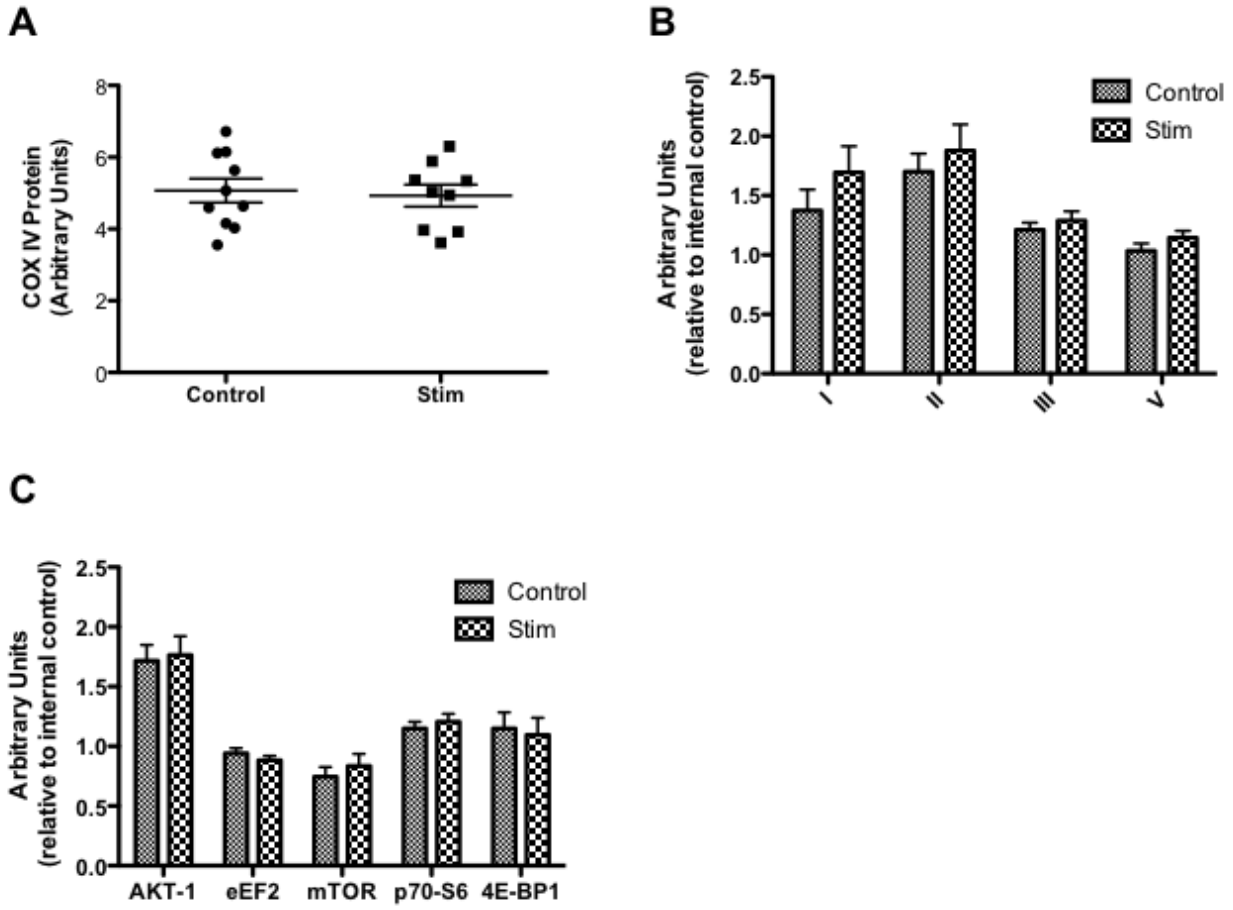
Human primary skeletal muscle cells (HSkMC) isolated from lean individuals were electrically stimulated 3 h/d for 3 d (Stim) with a protocol modified from Donnelly et al. (42). Fatty acid oxidation (FAO) was then measured 21 h after the last stimulation bout with a 3 h exposure to radiolabeled oleate [100μM] **(A-D)**. **(A)** Initial FAO trials (n=3, P=0.13). **(B)** FAO in two additional subjects (n=2, P=0.77). **(C)** FAO in all five trials (n=5, P=0.59). **(D)** FAO with outlier removed (n=4, P=0.10)

Figure 2.2. The effect of intermittent electrical stimulation on mitochondrial content in human primary skeletal muscle cells.



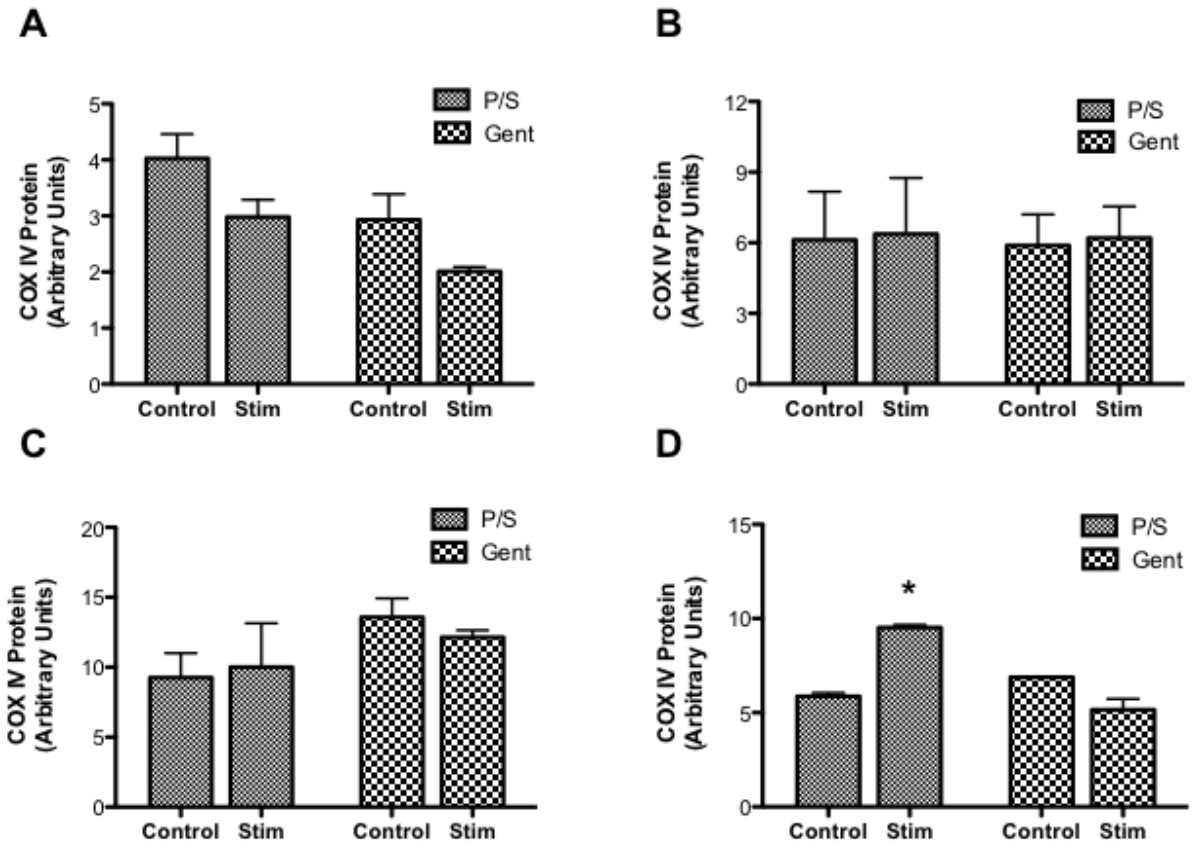
Mitochondrial content as assessed by Western blot in human primary skeletal muscle cells (HSkMC) isolated from lean individuals that were electrically stimulated 3 h/d for 4 d (Stim) as previously described (188) (**A-B**). (**A**) COX IV protein (Cell Signaling). (**B**) OXPHOS proteins (MitoSciences). Data represent mean ± SEM; n = 4 **(A)**, n = 4 **(B)**. *Different from Stim ($P < 0.05$).

Figure 2.3. Mitochondrial protein or selected proteins of the protein synthesis pathways in response to intermittent electrical stimulation in C2C12 cells.



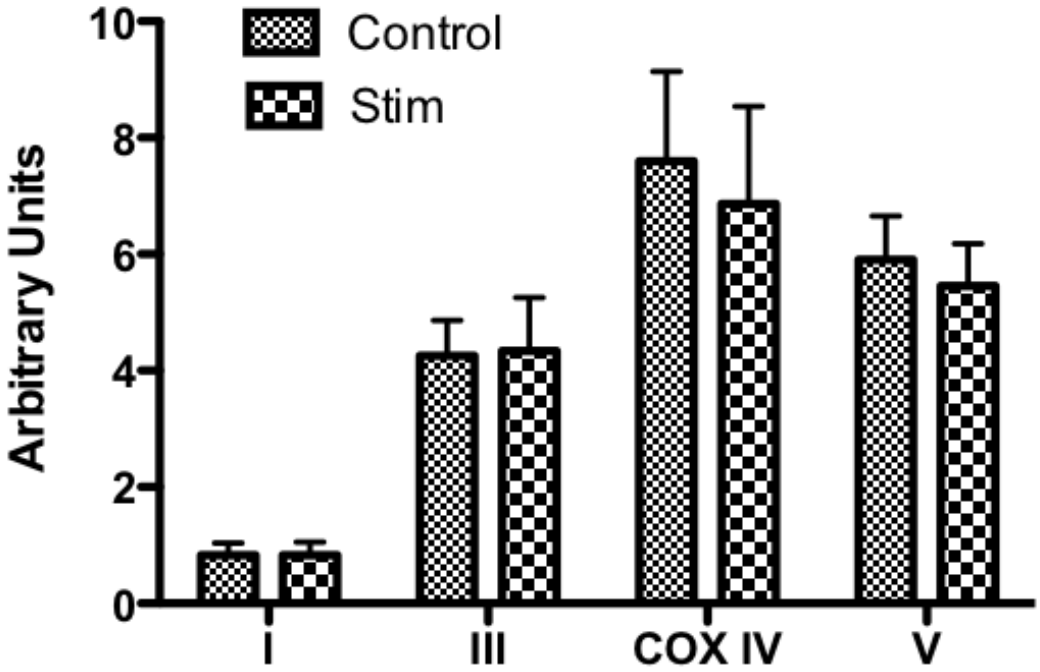
Indices of mitochondrial content and protein synthesis proteins were assessed by Western blot in C2C12 cells after electrical stimulation for 3 h/d for 4 d (Stim) as previously described (188) (**A-C**). (**A**) COX IV protein. (**B**) OXPHOS proteins (MitoSciences). (**C**) Western blot analysis of proteins involved in the protein synthesis pathway. Data represent mean ± SEM; n = 9-10 (**A**), n = 7 (**B**), n = 7 (**C**).

Figure 2.4. The effect of antibiotic on the ability of various intermittent electrical stimulation protocols to induce mitochondrial biogenesis in C2C12 cells.



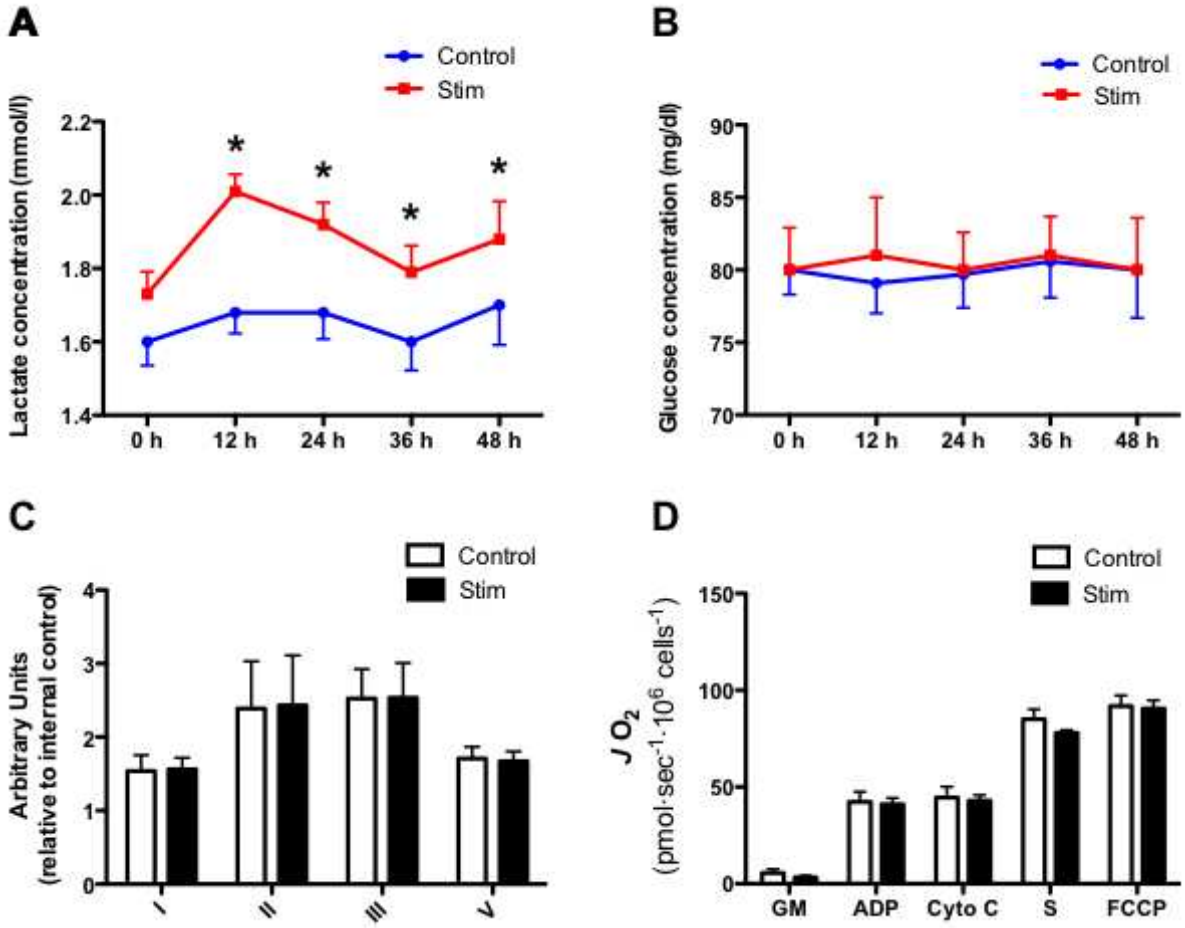
C2C12 cells were electrically stimulated for 3 h/d for 4 d in differentiation media containing the antibiotics penicillin-streptomycin (P/S) or gentamicin (Gent) and analyzed for COX IV protein **(A-D)**. C2C12 cells were electrically stimulated with protocols adapted from **(A)** Uguccioni et al. (188), (**B**) Burch et al. (28), (**C**) Donnelly et al. (42), or **(D)** Pinheiro et al. (145). Data represent mean ± SEM; n = 2 (**A & D**), n = 3 **(B** & **C**). *Different from Control-P/S ($P < 0.05$).

Figure 2.5. Mitochondrial protein in response to intermittent electrical stimulation in primary human myotubes.



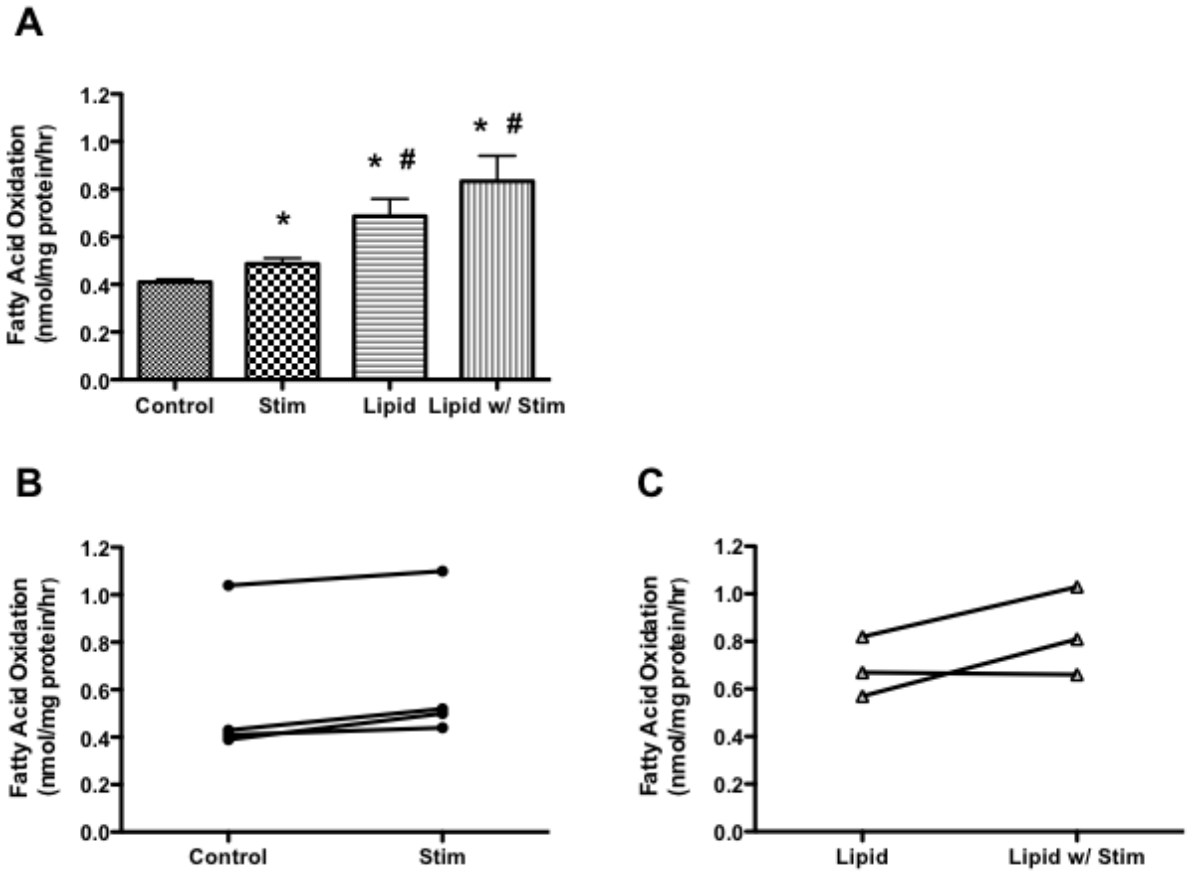
OXPHOS proteins (Complexes I, III, and V) and COX IV protein were assessed in primary human myotubes following electrical stimulation for 3 h/d for 4 d with a protocol modified from Pinheiro et al. (145). Data represent mean ± SEM; n = 4

Figure 2.6. The effect of continuous electrical stimulation for 48 h on human primary myotubes.



Human primary myotubes were exposed to continuous electrical stimulation for 48 h with a protocol modified from Nikolic et al. (131) **(A-D)**. Differentiation media was collected every 12 h for determination of **(A)** lactate and **(B)** glucose concentrations. **(C)** OXPHOS protein content was determined from cell lysates. **(D)** Rates of O_2 consumption in response to GM – glutamate [10mM] malate [2mM]; ADP [4mM]; Cyto C – cytochrome C [10µM]; S – succinate [10mM]; FCCP - Carbonyl cyanide p trifluoro methoxyphenylhydrazone [2µM]. Data represent mean ± SEM; n = 5 **(A, B)**, n = 4 **(C, D)**. *Different from respective control time point ($P < 0.05$).

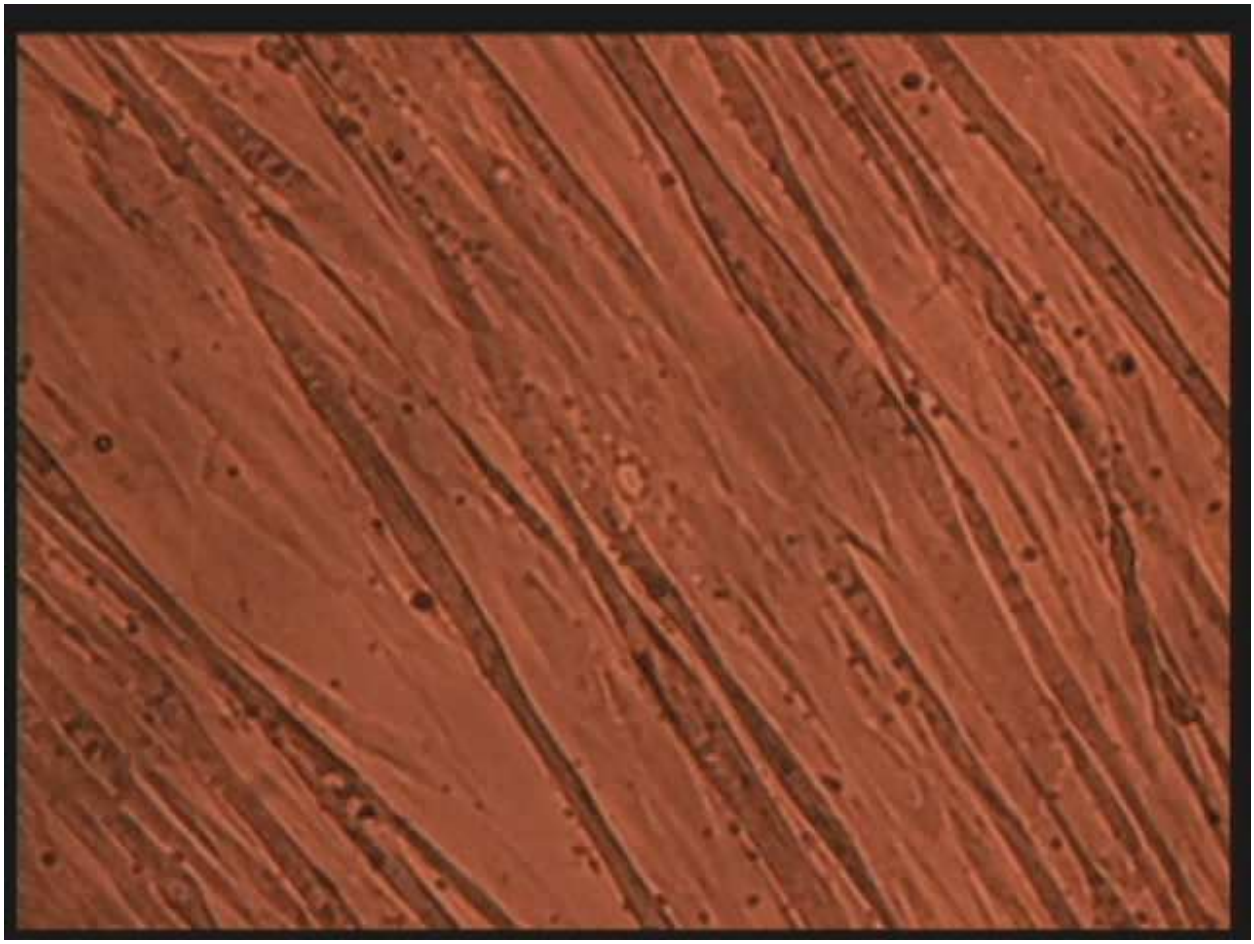
Figure 2.7. The effect of 48 h of lipid and/or continuous electrical stimulation on fatty acid oxidation in human primary myotubes



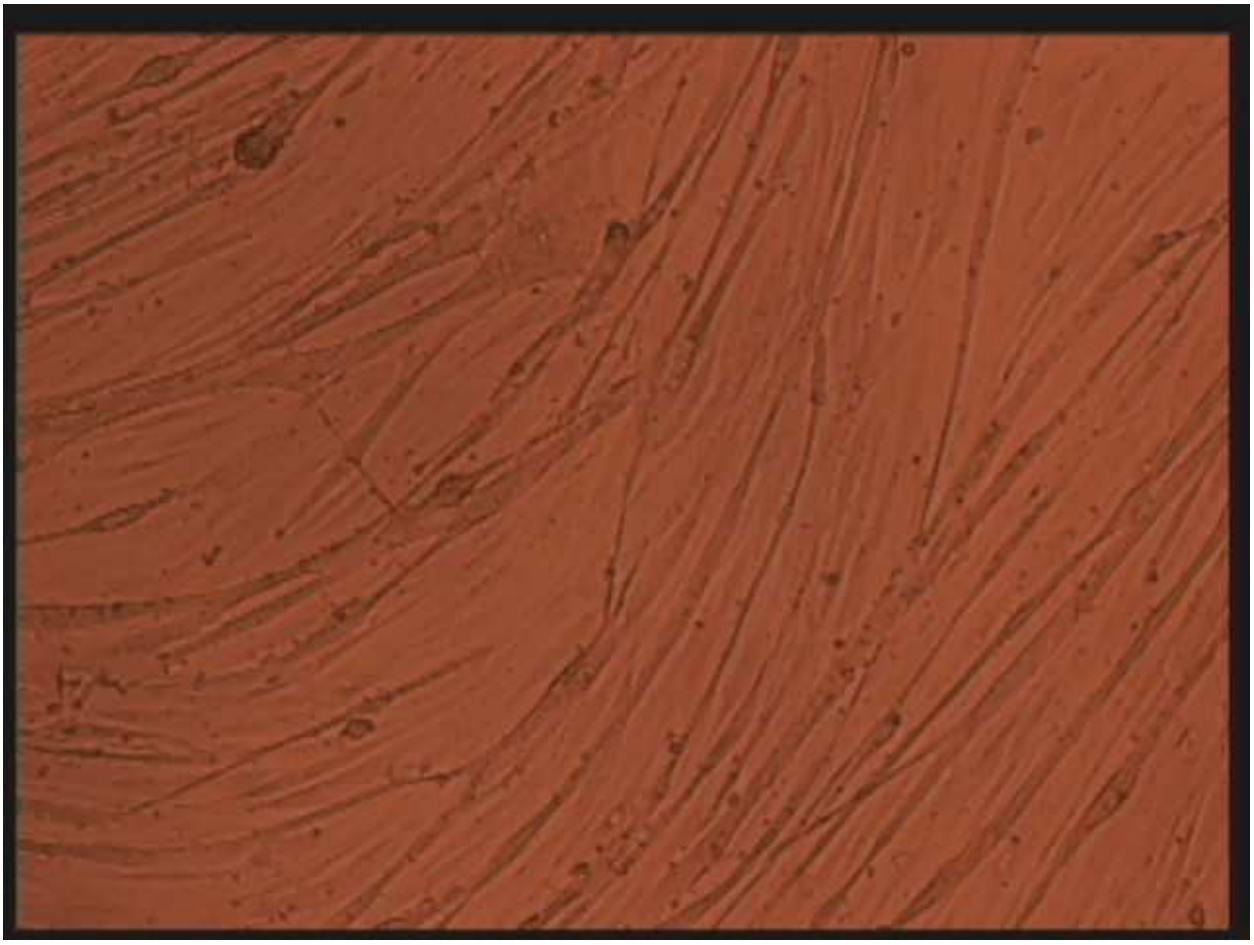
Human primary myotubes were exposed to continuous electrical stimulation for 48 h with a protocol modified from Nikolic et al. (131) without lipid (Stim) with lipid (Stim w/ Lipid), or lipid alone (Lipid) **(A)**. The 48 h lipid incubation consisted of a 1:1 oleate/palmitate mixture [100μM]. Fatty acid oxidation (FAO) was then measured 15 h after stimulation with a 3 h exposure to radiolabeled oleate [250μM] **(A-C).** Data represent mean ± SEM; n = 3-4 **(A)**, n = 4 **(B)**, n = 3 **(C)**. *****Different from Control ($P < 0.05$). $^{\#}$Different from Stim ($P < 0.05$).

Supplementary Materials

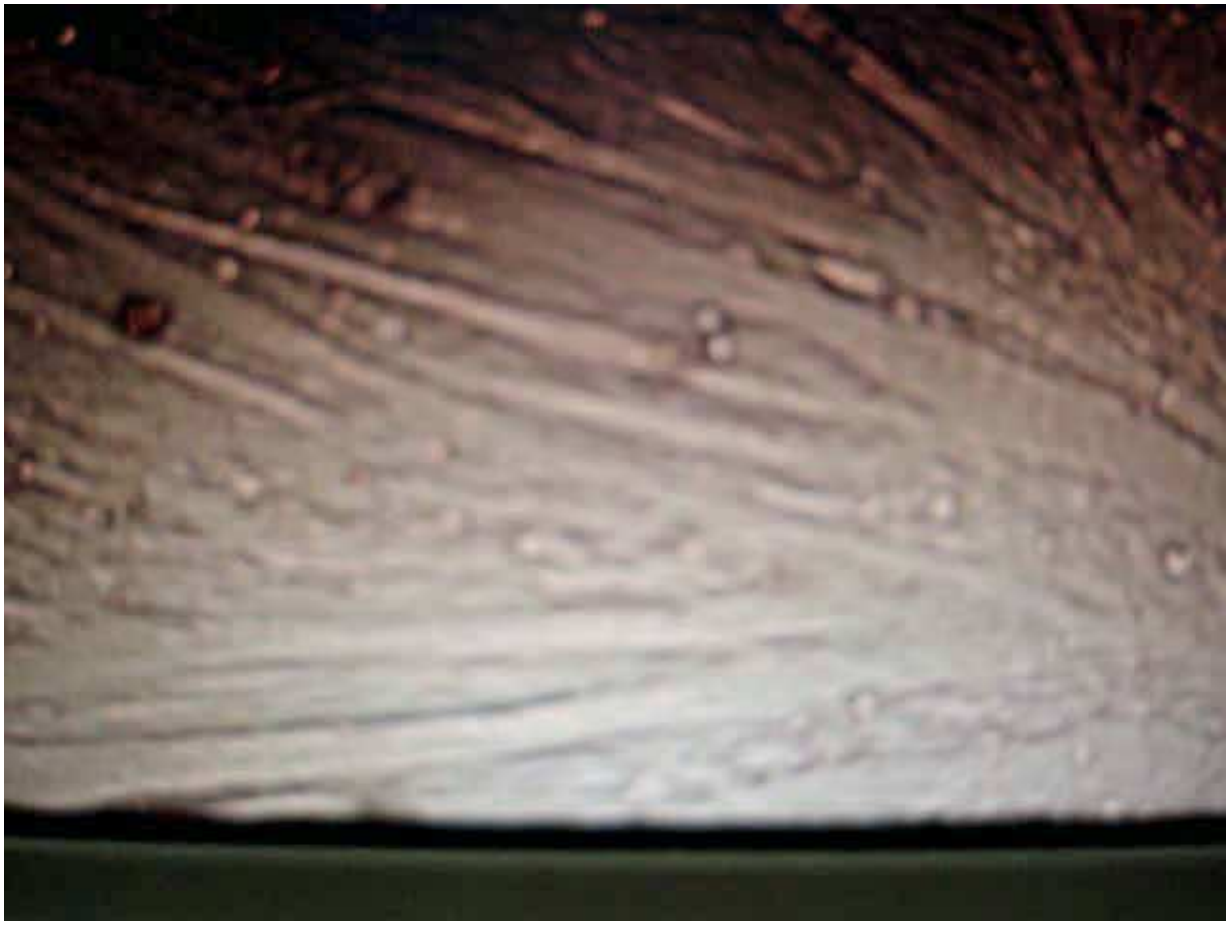
Video 2.1. Contracting human skeletal muscle myotubes with electrical pulses at 50 V 10 Hz (4 x 0.3 ms pulses delivered in 400 ms trains with 3.6 s recovery).



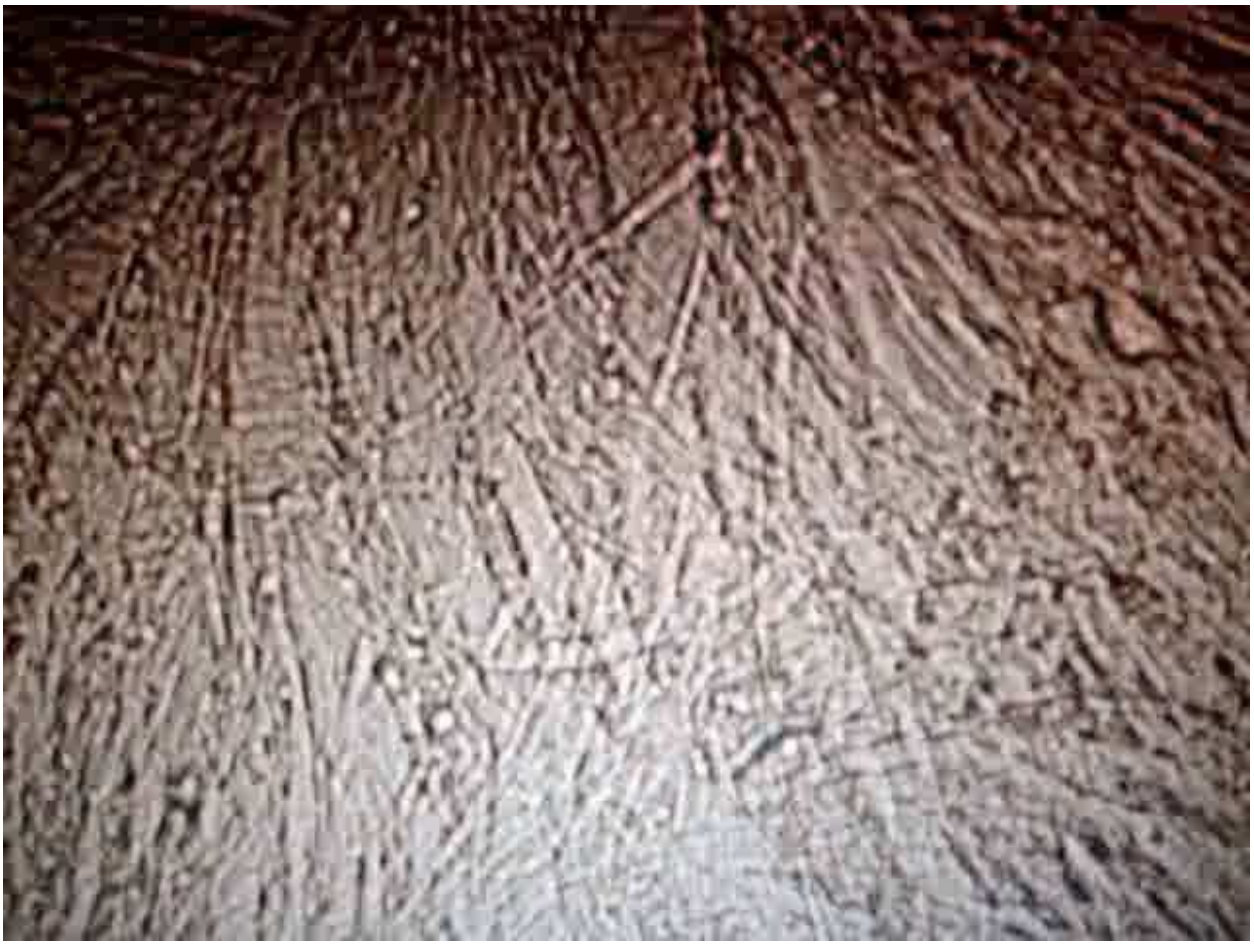
Video 2.2. Contracting human skeletal muscle myotubes with electrical pulses at 50 V 10 Hz (4 x 0.3 ms pulses delivered in 400 ms trains with 3.6 s recovery).



Video 2.3. Contracting C2C12 myotubes with electrical pulses at 50 V 10 Hz (4 x 0.3 ms pulses delivered in 400 ms trains with 3.6 s recovery).



Video 2.4. Contracting C2C12 myotubes with electrical pulses at 50 V 10 Hz (4 x 0.3 ms pulses delivered in 400 ms trains with 3.6 s recovery).



CHAPTER 3: THE EFFECT OF SUBSTRATE CHALLENGES ON METABOLIC FLEXIBILITY IN MYOTUBES DERIVED FROM LEAN AND SEVERELY OBESE DONORS

Abstract

Objective-Considerable debate exists whether alterations in skeletal muscle mitochondrial oxidative capacity and/or content play a causal role in the development of obesity. In addition, the skeletal muscle of severely obese individuals is considered to be metabolically inflexible to its nutrient environment. The purpose of this investigation was to determine whether decrements in oxidative capacity and metabolic inflexibility are retained in human skeletal muscle cell culture (HSkMC).

Research design and methods-Muscle biopsies from the vastus lateralis of young lean (22 ± 1 yr, BMI 22.9 ± 0.7 kg/m^2) and severely obese (26 ± 3 yr, BMI 40.7 ± 1.8 kg/m^2) men were obtained for the culturing of HSkMC. Measures of respiratory capacity were performed in permeabilized and intact primary myotubes along with citrate synthase activity (CS), COX IV and OXPHOS protein content, and media measures of glucose and lactate after 24 h incubations with BSA (Con), oleate/palmitate (O/P), galactose (Gal), or pyruvate (Pyr).

Results-No differences in mitochondrial respiratory function or content were observed between lean and severely obese subjects with or without the addition of oxidizable substrates. Both groups significantly increased the capacity to respire in response to all three oxidizable substrates and increased markers of mitochondrial content to a similar extent. Both groups also demonstrated metabolic flexibility by decreasing reliance on glucose in the presence of oxidizable substrates.

Conclusions-The results herein suggest that reductions in mitochondrial respiratory capacity/content and metabolic inflexibility, if present during obesity, are most likely secondary to the initial manifestation of obesity.

Introduction

Severe obesity is a condition characterized by a body mass index (BMI) $\geq$ 40.0 kg/m^2, expanded adipose stores, and insulin resistance. Obesity is clearly the result of a chronically positive energy balance, however, the degree to which obese individuals accumulate lipid stores may also be due to an increased propensity to store rather than oxidize lipid. The skeletal muscle of severely obese individuals has a dampened ability to completely oxidize fatty acids (88, 97) and this inability to oxidize fatty acids is associated with a positive lipid balance (5, 182) that may promote weight gain.

In addition to depressed rates of skeletal muscle fatty acid oxidation (FAO), severely obese individuals appear to be resistant to stimuli affecting substrate selection. For example, the imposition of a high fat diet (HFD) increases FAO (10) and the expression of FAO regulating genes (22) in lean individuals whereas obese individuals exhibit virtually no change. The inability of the obese individual to increase FAO in the face of a lipid challenge is part of a larger phenomenon known as "*metabolic inflexibility*". Originally, David Kelley's group coined the term "metabolic flexibility" when they reported that, in response to insulin stimulation, obese skeletal muscle was lacking "the capacity to switch from predominately lipid oxidation and high rates of fatty acid uptake during fasting conditions to the suppression of lipid oxidation and increased glucose uptake oxidation, and storage under insulin-stimulated conditions" (96).

The observation that obese skeletal muscle fails to alter fuel selection in the face of a lipid challenge is interesting, however, whether the inability to upregulate FAO is a trait of skeletal muscle *per se* or whether this is a secondary consequence of other biological factors present in the obese, but not the lean population cannot be determined from *in vivo* measurements. For example, obese individuals possess substantially larger adipose stores and as such the release of free fatty acids (FFA) into circulation is substantially greater (125). Elevations in FFA by way of lipid infusion (21) or short-term starvation (83) have been shown to induce insulin resistance and reduced metabolic flexibility even in the absence of obesity (21, 83). Furthermore, adipose tissue is not an inert storage depot, as it is capable of secreting numerous factors known to alter metabolism (134, 136). It has also become widely accepted that in obesity, adipose tissue recruits immune cells and aids in mediating a low-grade inflammatory process (4, 34, 132) known to induce insulin resistance. Any combination of these factors makes the *in vivo* environment in obesity difficult for studying the underlying mechanisms of metabolic flexibility. On the other hand, human primary skeletal muscle cell culture (HSkMC) provides a controlled environment for studying skeletal muscle metabolic flexibility as it lacks the endocrine environment present in obesity, yet retains the phenotypic traits of the donor skeletal muscle (15).

The Western diet, which in large part is responsible for the obesity epidemic (168, 174) is not only high in fat, but contains excess kilocalories from carbohydrate (CHO) as well (7). The purpose of the current investigation was to compare the effects of lipid or CHO overexposure in myotubes cultured from lean and severely obese skeletal muscle. To that end we incubated human primary skeletal muscle cells in either lipid (oleate/palmitate) or CHO (galactose or pyruvate) and measured metabolic flexibility in terms of changes in mitochondrial content and

respiration in permeabilized or intact cells. The results contained herein suggest that reductions in mitochondrial respiratory capacity/content and metabolic flexibility present in obesity may be secondary to the initial manifestation of obesity and the altered regulation of the intracellular and systemic environment present in obesity rather than an inherent trait of skeletal muscle.

Methods

Human subjects. Healthy, sedentary male subjects (18-40 yrs) that were lean (BMI 22.9 ± 0.7 kg/m^2, n=10) or obese (BMI 40.7 ± 1.8 kg/m^2, n=10) were recruited from the faculty and student population of East Carolina University. Exclusion criteria included smoking, the presence of diabetes, or heart disease, and taking any medications known to alter metabolism. Subject characteristics are described in Table 3.1. The Medical Center Institutional Review Board at East Carolina University approved all procedures in this study and all participants signed a written informed consent.

Primary Human Skeletal Muscle Cell Culture (HSkMC). Following an overnight fast (~12 hrs), a skeletal muscle biopsy was obtained from the vastus lateralis by the percutaneous needle biopsy technique (15, 127). The isolation and culturing of human primary skeletal muscle cells from biopsies was performed as previously described (36). On day 7 of differentiation, cells were incubated for 24 h in differentiation media supplemented with either 0.1% bovine serum albumin (BSA) (Control), 100 μM oleate:palmitate (1:1 ratio) bound to 0.1% BSA plus 2 mM carnitine (O/P), 10 mM galactose (Gal), or 10 mM pyruvate (Pyr) after which the cells were harvested for respirometry experiments. Separate aliquots of the same passage number were

grown and treated similarly, then harvested for the analysis of protein content and citrate synthase activity.

Respirometry experiments: Permeabilized Cells. Myotubes were permeabilized for respiration experiments as previously described (106). Myotubes were washed with PBS and lifted from culture flasks with 0.05% trypsin EDTA. This reaction was neutralized by adding 10% FBS to the cell suspension and centrifuged for 10 min at 1000 rpm at room temperature. The cell pellet was then resuspended in growth media, counted using a hemocytometer, centrifuged again for 10 min, and re-suspended in room temperature respiration buffer (130 mM sucrose, 60 mM potassium gluconate, 1 mM EGTA, 3 mM magnesium chloride, 10 mM potassium phosphate, 20 mM HEPES, 0.1% BSA; pH 7.4). The cells were then treated with 3 μg/10^6 cells/ml digitonin (i.e., a mild, cholesterol-specific detergent) for 5 min at 37° C on an orbital shaker. Following permeabilization, myotubes were washed by centrifugation at 1000 rpm for 5 min to remove endogenous substrates. The cells were then resuspended in respiration buffer at a concentration of (1.5×10^6 cells per 2 ml) and loaded into a respiration chamber (Oroboros Oxygraph-2K, Oroboros Instruments Corp., Innsbruck, Austria). To avoid potential limitations due to oxygen availability, all experiments were conducted after the oxygraph chamber was hyperoxygenated to an oxygen concentration of ~300 nmol/ml. Side-by-side experiments under control, oleate/palmitate, galactose, and pyruvate conditions were performed on cells from each subject. Oxygen consumption was normalized to total protein (BCA assay; Pierce Biotechnology, Rockford, IL).

For the permeabilization experiments, substrate concentrations were chosen based upon the methods of Pesta & Gnaiger (138) known to elicit maximal rates of respiration in human cells. The concentration and sequence of substrate titrations are found in Table 3.2.

Respirometry experiments: Live Cell. Myotubes were harvested as described above and resuspended in fresh differentiation media identical to the media the cells were originally incubated in for 24 h (BSA, O/P, Gal, or Pyr) at a concentration of (2.0×10^6 cells per 2 ml) and loaded into the respiration chamber (Oroboros Oxygraph-2K, Oroboros Instruments Corp., Innsbruck, Austria). Oxygen consumption was measured during basal respiration and after the addition of 5 μM FCCP. Data was normalized to cell count.

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 1 h, and centrifuged at 20,000 g for 30 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 fluoride (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). Membranes were incubated for 1 h 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. Citrate synthase activity was determined using a standard assay kit (Sigma CS0720, St. Louis, MO), which colorimetrically measures the reaction rate between acetyl coenzyme A and oxaloacetic acid.

Glucose and Lactate. Media was collected after 24 h incubations and immediately frozen at -80° C for the subsequent determination of glucose and lactate. Glucose and lactate were determined by oxidation reaction (YSI model 2300 Stat Plus; Yellow Springs Instruments, Yellow Springs, OH). Glucose disappearance was calculated as the glucose concentration at the end of the incubation period subtracted from the concentration in the stock differentiation media. Measures of glucose disappearance and media lactate were then normalized to cell count.

Statistical Analyses. Data are presented as mean ± SEM. Statistical analyses were performed using t-tests for subject characteristics, 1-way ANOVA with Student-Newman-Keuls or 2-way repeated measures ANOVA with Bonferroni post hoc test where appropriate for the analysis of significance among groups. The level of significance was set at $P < 0.05$.

Results

Subject characteristics. Subject characteristics are presented in Table 3.1. By design, the obese individuals were heavier and had a significantly higher body mass index (BMI) (22.9 ± 0.8 kg/m^2, 40.7 ± 1.8 kg/m^2) than their lean counterparts ($P < 0.0001$). Fasting blood glucose was similar between groups; however, corresponding insulin values were significantly elevated in the obese subjects ($P = 0.001$). As, expected, HOMA-IR values were also significantly elevated in obese subjects, suggesting an insulin resistant phenotype ($P = 0.004$).

Obesity does not impact mitochondrial content or respiratory capacity within primary human myotubes. To assess whether mitochondrial content in myotubes from lean and obese differed, we measured citrate synthase activity (CS), COX IV protein, and proteins of the OXPHOS cocktail (MitoSciences). These measures of mitochondrial content were selected as they represent two distinct compartments, the TCA cycle and the electron transport chain. CS, COX IV, or OXPHOS proteins did not differ between lean and obese under control conditions (Figures 1A-B).

To determine whether mitochondrial respiratory capacity differed in skeletal muscle cells prepared from lean and obese we assessed respiration in digitonin-permeabilized primary human myotubes. In order to assess the capacity for electron transfer within the entire system, a comprehensive protocol was designed in which saturating concentrations of substrates directed at complex-I, complex-II and β-oxidation were added in the presence of ADP (138) (Table 3.2). Utilizing this protocol, respiratory capacity was not different in myotubes from lean and obese (Figure 3.1C). Although not significant, the response to succinate (P = 0.23) and FCCP (P = 0.31) tended to be higher in lean cells, suggesting a possible divergent rate of electron transport at the level of complex-II between groups. To address this possibility, succinate supported respiration was assayed in the presence of rotenone under basal (S_4), maximal-ADP (S_3) and uncoupled (FCCP) conditions (Figure 3.1D). Once again, no significant differences were observed between lean and obese myotubes.

Acute exposure to oxidizable substrates increases mitochondrial content in lean and obese to a similar extent. There were no differences in the absolute amount of OXPHOS, COX IV protein, or CS activity between lean and obese after 24 h substrate incubations (data not shown). There were, however, differences in the lean/obese responses to the incubations. Complex I protein

increased in both groups in response to O/P (Figure 3.2A) and galactose (Figure 3.2B) (all $P < 0.001$) in contrast to pyruvate where the lean increased ($P = 0.01$) but the obese did not ($P = 0.17$) (Figure 3.2C). Complex II and III protein increased in the lean group in response to O/P (both $P = 0.01$) and galactose (both $P < 0.05$) but neither changed in the obese group (Figures 3.2A-B). Both groups tended to increase CS in response to O/P (lean $P = 0.08$, obese $P = 0.04$) (Figure 3.2A) and galactose (both $P < 0.05$) (Figure 3.2B). Pyruvate incubation did not change Complex II or III protein or citrate synthase in either group (Figure 3.2C).

Acute exposure to oxidizable substrates increases the capacity to respire in lean and obese to a similar extent. There was a main effect for all three substrates (O/P, Gal, Pyr) to increase palmitoyl-carnitine malate supported respiration in the presence of ADP (PCM_3) ($P < 0.01$) and FCCP-stimulated respiration ($P < 0.01$) with no effect of group (Figures 3.3A-C). To demonstrate the collective effect of acute oxidizable substrate incubations during our respiratory protocol (Table 3.2), lean and obese groups were pooled (Figure 3.3D). Incubation with O/P, Gal, or Pyr significantly increased palmitoyl-carnitine malate (ADP), glutamate (G), succinate (S), and uncoupled respiration (FCCP) in the presence of ADP ($P < 0.01$).

Myotubes from lean and obese individuals increased the capacity to respire to the same extent when challenged with an acute 24 h exposure to oxidizable substrates (O/P, Gal, Pyr). We next sought to determine whether this increased capacity to respire translated into functional, real-time increases in oxygen consumption. In this set of experiments we utilized intact, rather than permeabilized myotubes. Compared to control conditions, basal respiration increased after Pyr incubation ($P < 0.05$). Respiration also tended to be elevated after O/P and Gal incubations, however, the difference did not reach significance (Figure 3.4A). The addition of FCCP increased respiration under all conditions, but was significantly elevated above FCCP control

when challenged with O/P, Gal, or Pyr ($P < 0.05$) (Figure 3.4A). Basal ($P = 0.09$) and FCCP-induced respiration ($P = 0.06$) tended to be higher in the cells prepared from obese subjects under control conditions when only glucose was available (Figure 3.4B).

To confirm that myotubes from lean and obese individuals increased their reliance on oxidative metabolism in response to lipid and CHO challenges, media lactate concentration and glucose disappearance were measured. As expected, glucose disappearance (Figures 3.5 A-B) and media lactate concentration (Figures 3.5 C-D) were lower for each substrate tested compared to control conditions. Although significance was not achieved, obese cells tended to utilize less glucose (Figures 3.5 A-B) and produce less lactate (Figures 3.5 C-D) under all conditions compared to lean myotubes, suggesting an increased reliance on oxidative metabolism in the obese cells.

Discussion

In the present study, measures of OXPHOS protein content, citrate synthase activity, and cellular respiration were not different between myotubes derived from lean and obese subjects. Furthermore, *metabolic flexibility* as determined by the ability to adapt to oxidizable substrates was not substantially different between groups. Collectively, the present findings do not support a role for inherent detriments in mitochondrial oxidative capacity (often referred to as "mitochondrial dysfunction") as a primary "cause" of obesity.

Mitochondrial content (85, 95, 97, 152) and FAO (14, 85, 88, 97, 183) tend to be reduced in the skeletal muscle of obese individuals. However, from these *in vivo* studies it is difficult to determine whether obesity precedes decrements in FAO or whether decrements in FAO precede obesity. Obesity may take years to develop and is characterized by numerous endocrine

abnormalities that are likely the result of lifestyle choices. HSkMC is thought to retain the underlying phenotype of the parent donor thereby providing a potential avenue to study obesity in individuals prone to obesity before it develops. For that reason, human primary skeletal muscle cell culture (HSkMC) was utilized in this study. Data from human primary myotubes in the field of obesity research have thus far been contradictory. In a previous investigation, human primary cells isolated from severely obese women (BMI = 45.3 ± 1.4) displayed significant reductions in mtDNA (27%), COXIV protein (35%), and TFAM protein (43%) (36). In contrast, HSkMC isolated from severely obese men (BMI = 39.0 ± 2.0) displayed no decrease in mtDNA, COXIV protein, or CS (23), findings that are in agreement with the current investigation (Figures 3.1A-B). The discrepancies in the cell culture studies may originate from differences in gender and adiposity although the exact mechanisms are not apparent.

In the current investigation under control conditions respiratory capacity was similar between lean and obese (Figures 3.1C-D), which coincides with similar mitochondrial content between groups (Figures 3.1A-B). In human primary skeletal muscle myotubes, Boyle et al (23) also reported no difference in respiratory capacity in lean and severely obese individuals. Collectively, these data tend to suggest that skeletal muscle oxidative capacity in lean and obese individuals may not be inherently different in skeletal muscle myotubes when independent of metabolic influences *in vivo*.

The response to a metabolic challenge, such as an increase in lipid availability, may be a stronger determinant in the development of obesity than initial oxidative capacity. In response to a high fat diet, lean individuals rapidly increase fatty acid oxidation to match nutrient availability whereas obese individuals do not (10, 182). Boyle et al (23) extended these findings to human primary skeletal myotubes by demonstrating that, when exposed to a 24 h lipid challenge,

myotubes cultured from lean individuals upregulate the capacity to oxidize fatty acids whereas their obese counterparts do not respond. The ability of the lean group to appropriately match nutrient oxidation with nutrient availability is known as *metabolic flexibility* (96). To expand upon the findings of Boyle et al (23) and to investigate the effects of other sources of excess kilocalories in the Western diet, we sought to determine whether skeletal muscle cells from severely obese individuals were also unresponsive (*metabolically inflexible*) when challenged with excess CHO in the form of either galactose or pyruvate.

In contrast to skeletal muscle tissue, which relies almost exclusively on oxidative phosphorylation to meets its energetic needs in the basal state, cells grown in culture derive a considerable amount of ATP via glycolysis (150). Replacing glucose with galactose in the culture media has previously been shown to induce widespread alterations in mitochondrial content, morphology and oxidative capacity, presumably as a consequence of increased reliance on oxidative phosphorylation within the galactose grown cells (2). In addition, supraphysiological pyruvate incubations have been shown to increase mitochondrial content in immortalized cell lines (143, 204). In order to determine any potential differences in adaptability between lean and obese cells, myotubes were incubated for 24 hours in the presence of three different oxidizable substrates (oleate/palmitate, galactose, pyruvate).

Interestingly, metabolic flexibility was retained in HSkMC isolated from severely obese individuals in response to not only excess CHO but lipid as well: CS and OXPHOS protein (Figures 3.2A-C) and permeabilized respiration (Figures 3.3A-C) increased and glucose utilization decreased (Figures 3.5A-B) in response to lipid or CHO incubations in both the lean and the severely obese groups. These results are in opposition to those reported by Boyle et al (23), in which myotubes from severely obese individuals failed to increase the capacity to

oxidize fatty acids in response to a 24 h lipid incubation. The contrast in outcomes between the previous and present investigation are likely the result of differences in methodologies as each study utilized an established, yet different permeabilization protocol, (105) and (106) respectively. Boyle et al (23) carried out cell permeabilization directly in the oxygraph chamber (we permeabilized prior to placing the cells in the oxygraph) and utilized digitonin concentrations 2-3 fold higher and substrate concentrations 2-3 fold lower than those reported herein. We also hyperoxygenated the oxygraph chamber whereas Boyle et al did not. The robust ADP response in all conditions combined with the absence of a cytochrome C response makes us confident that myotubes in the current investigation were appropriately permeabilized. The exact cause of the difference in results between studies remains uncertain.

Several investigators have demonstrated that metabolic flexibility is absent in myotubes derived from obese individuals when exposed to lipid (37, 99), or grown in galactose (2), however the obese subjects in these previous studies were also diabetic. Metabolic disease tends to operate on a continuum with lean individuals being the most healthy, diabetics the least, and obese falling somewhere in-between. In studies investigating the response to metabolic stimuli (lipid, galactose, acetate, insulin) in myotubes derived from lean, obese, and diabetic individuals, lean and obese groups tend to respond in a similar fashion, whereas the diabetic response is severely inhibited (2, 38, 61-63, 133, 200). It should be noted that in the majority of these investigations, the obese group is not severely obese, however, the findings in the current investigation suggest that metabolic flexibility in the severely obese group remains intact.

Although the present findings demonstrate the capacity to respire and the rate of oxygen consumption do not differ between groups with the addition of oxidizable substrates, these measures give no indication of whether glucose or fatty acids were utilized to meet those oxygen

demands. Therefore, some degree of metabolic inflexibility cannot be ruled out. In the present study, the obese subjects were significantly more insulin resistant as indicated by an increased HOMA value (Table 3.1). Galgani et al (57) has attributed the *in vivo* metabolic inflexibility in obese and diabetic individuals described by Kelley et al (96) primarily to insulin resistance, as normal metabolic flexibility is exhibited when metabolic flexibility is normalized to glucose uptake. Although no insulin was utilized in the present investigation there were differences in glucose metabolism between groups. The obese group took up less glucose and produced less lactate (Figures 3.5A & C), yet consumed more oxygen during basal conditions (Figure 3.4A). Exposure to oxidizable substrates did not further enhance basal oxygen consumption in the severely obese group whereas respiration tended to increase in the lean group (Figure 3.4A). These findings may suggest that under basal conditions the lean group was metabolically flexible, whereas the obese was not. However, when energy demand was increased through the addition of FCCP to the oxygraph chamber, the obese group increased oxygen consumption well beyond that of glucose only supported respiration in the presence of lipid, galactose, and pyruvate, demonstrating metabolic flexibility remained intact.

Is metabolic flexibility an inherited or a developed trait? Gaster et al (61) previously demonstrated that metabolic flexibility remains intact in myotubes from obese and diabetic groups in response to fatty acids and insulin. Although the differences in the responses between groups in the Gaster et al (61) investigation were not significantly different, there were clear stepwise reductions in the magnitude of the responses from lean to obese to diabetic. The obese subjects in the current investigation were young (26 ± 3 yr), healthy, and likely not far into the progression of metabolic disease, which may have contributed to their ability to remain metabolically flexible. Following standardized weight loss protocols, post-diabetic individuals

have been shown to improve *in vivo* insulin sensitivity similar to their lean counterparts (2) or no longer exhibited diabetes by measures of fasting blood glucose and hemoglobin A1C (38). However, in each investigation, impairments in mitochondrial biogenesis (38) or oxygen consumption rate (2) were still apparent in myotubes established from muscle biopsies after weight loss. It is currently unknown whether metabolic flexibility is restored after weight loss in severely obese individuals that are older and further into the disease progression. Future investigations should determine the longitudinal effects of weight loss, exercise, and dietary macronutrient variations on the effect of HSkMC substrate selection and metabolism.

Conclusion. The results of the current investigation do not support a causal role for reductions in oxidative capacity to precede the obese phenotype. There were no substantial differences in citrate synthase activity, COX IV protein, or OXPHOS protein in myotubes derived from lean and obese individuals at baseline or in response to an acute exposure to oxidizable substrates nor were there any differences noted in respiratory capacity measured in permeabilized myofibers or in intact cells. Incubation of myotubes with oxidizable substrates decreased glucose utilization and favored a more oxidative phenotype in lean and severely obese, indicating metabolic flexibility was intact in both groups. Taken together these results suggest that the decrements in oxidative capacity associated with obesity are likely secondary to the effects of obesity, such as expanded adipose stores, the inability to regulate free fatty acids during insulin stimulation, insulin resistance, and physical inactivity, and not a defect inherent to skeletal muscle.

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.

Table 3.1. Characteristics of the study groups.

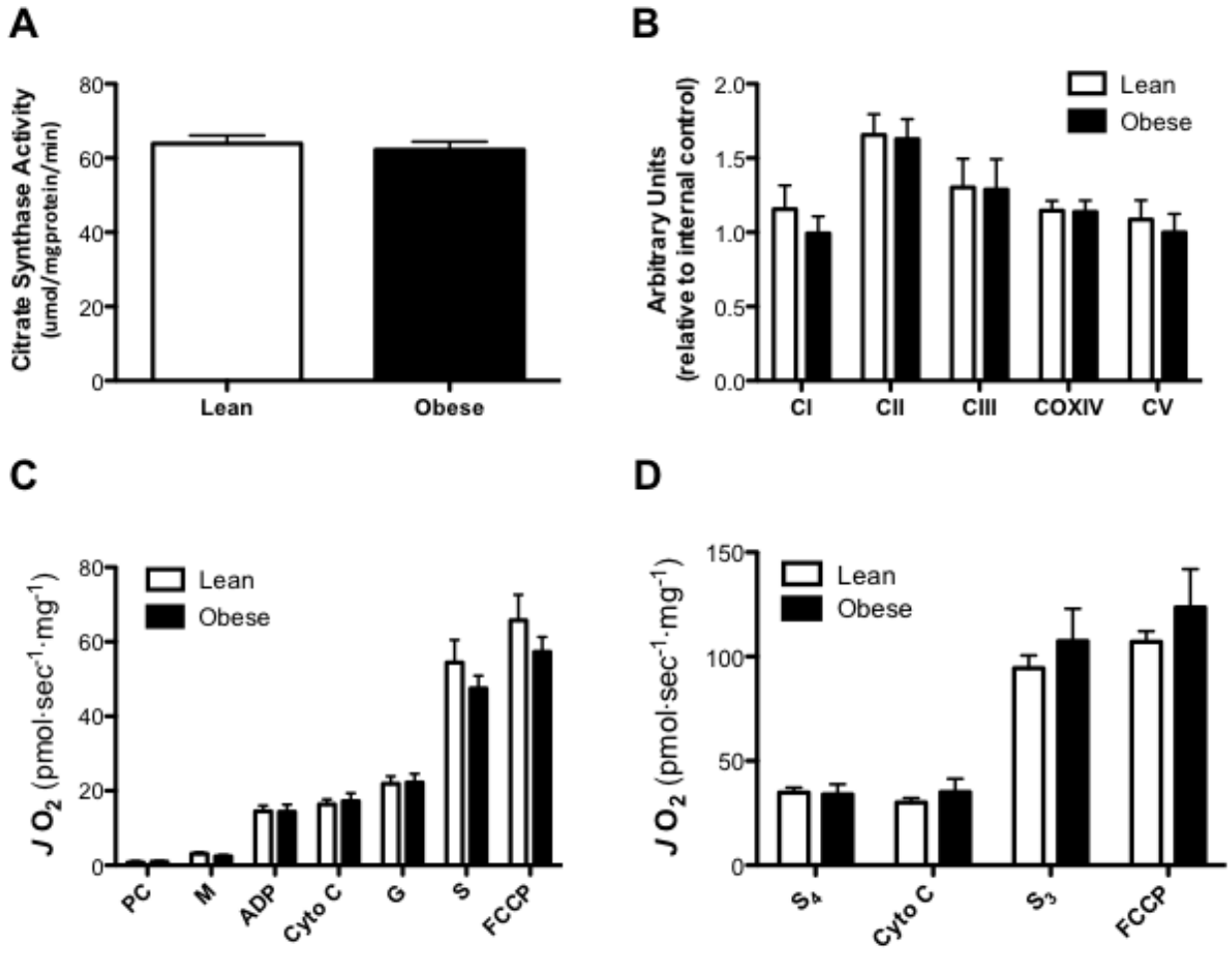
	Lean (n = 10)	Obese (n = 10)
Age [yr]	22 ± 1	26 ± 3
BMI [kg/m^2]	22.9 ± 0.8	40.7 ± 1.8*
Body fat [%]	21.6 ± 2.2	38.9 ± 1.4*
Fasting plasma glucose [mg/dL]	84.5 ± 2.7	86.8 ± 2.7
Fasting plasma insulin [μU/mL]	3.5 ± 0.8	14.5 ± 1.9*
HOMA-IR [$(mM*mU/L)^2$]	0.8 ± 0.2	3.1 ± 0.4*

Data are mean ± SEM. BMI, body mass index, * Different from lean ($P < 0.05$)

Table 3.2. Respirometry protocol for permeabilized cells

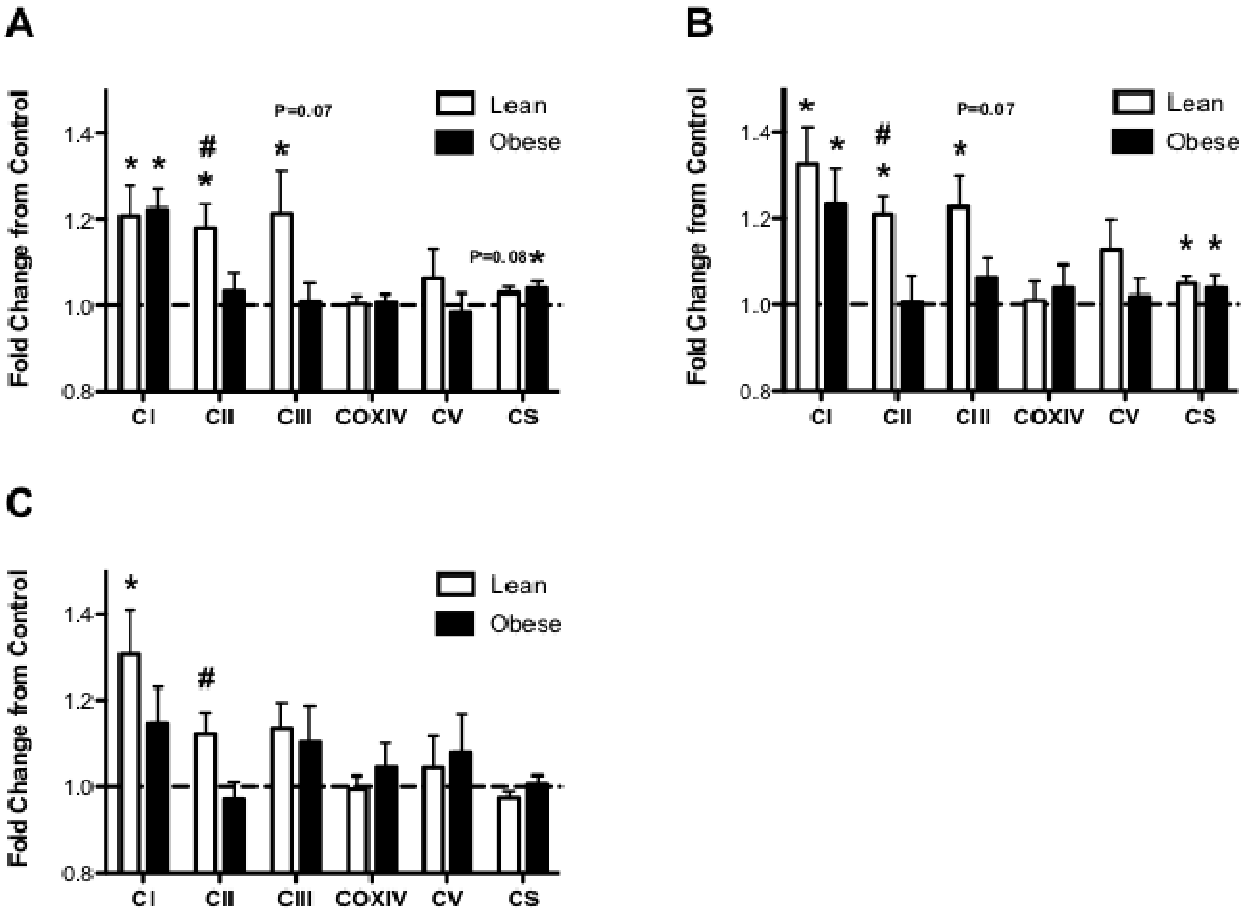
Step	Substrate	Notation	Concentration
1	Respiration Buffer		3 μg/10^6 cells/ml
2	Palmitoyl carnitine	PC	25 μmol l^{-1}
3	Malate	M	2 mmol l^{-1}
4	ADP	D	4 mmol l^{-1}
5	Cytochrome C	Cyt C	10 μmol l^{-1}
6	Glutamate	G	10 mmol l^{-1}
7	Succinate	S	10 mmol l^{-1}
8	FCCP	FCCP	2 μmol l^{-1}

Figure 3.1. Mitochondrial content and respiratory capacity in primary human myotubes from lean and obese individuals under control conditions.



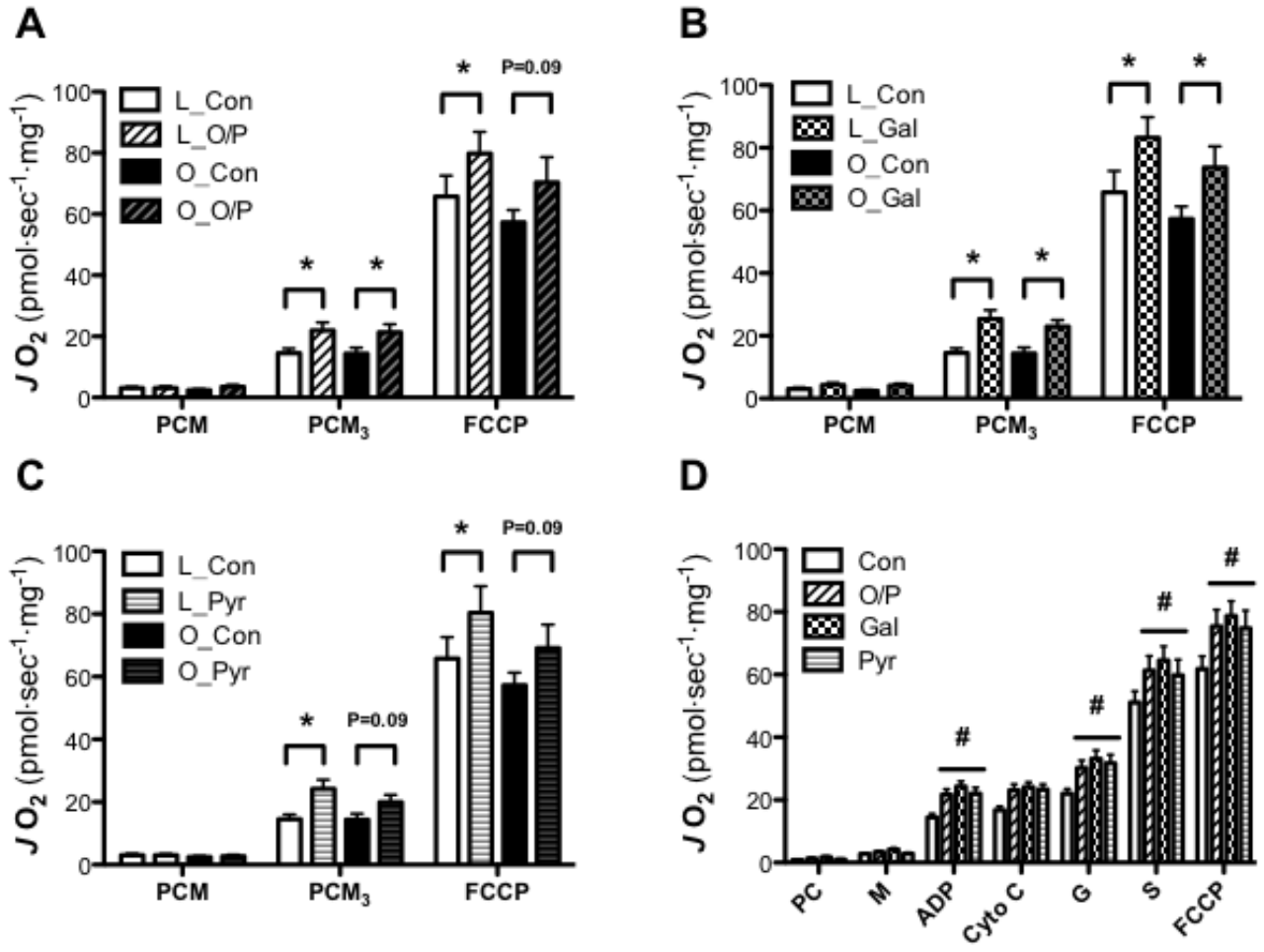
Mitochondrial content and oxygen consumption was assessed in human primary myotubes prepared from vastus lateralis muscle of lean and obese subjects (**A-D**). (**A**) Citrate synthase activity, expressed as μmol citrate/mg protein/min. (**B**) Western blot analysis of mitochondrial OXPHOS (MitoSciences) and COX IV protein prepared from cell lysate. (**C**) Rates of O_2 consumption in response to PC – palmitoyl carnitine [25μM]; M - malate [2mM]; ADP [4mM]; Cyto C – cytochrome C [10μM]; G – glutamate [10mM]; S – succinate [10mM]; FCCP - Carbonyl cyanide p trifluoro methoxyphenylhydrazone [2μM]. (**D**) Rates of O_2 consumption in the presence of succinate [10mM] plus rotenone [1μM], under basal (S_4), ADP-stimulated (S_3), and uncoupled (FCCP) conditions. Data represent mean ± SEM; n = 10 (**A**), n = 9-10 (**B**), n = 9-10 (**C**), n = 4-5 (**D**)

Figure 3.2. Fold change in citrate synthase activity (CS), COX IV, and OXPHOS protein in primary human myotubes from lean and obese individuals.



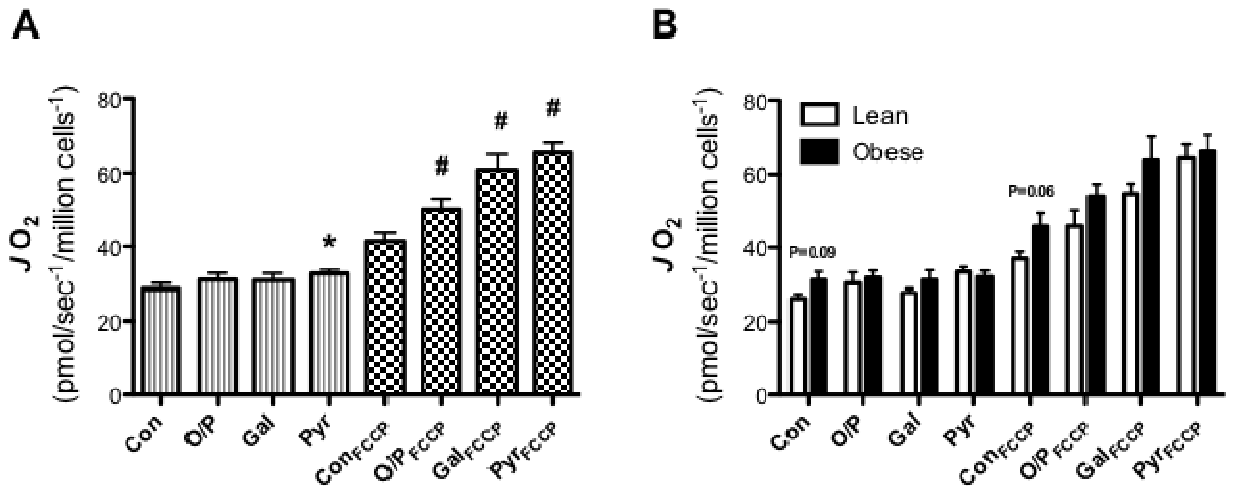
Mitochondrial content was determined in fully differentiated myotubes that were incubated for 24 hours in the presence of 0.1% BSA (Control), 100 μM oleate/palmitate **(A)**, 10 mM galactose **(B)** or 10 mM pyruvate **(C)**, all of which were added directly to the differentiation media. OXPHOS (Complexes I, II, III, and V) protein, COX IV protein, and citrate synthase activity (CS) were determined in myotubes from lean and obese and expressed as fold change from respective BSA control. Data are fold change normalized to control and represent mean ± SEM; n = 9-10 (**A-C**). *Different from BSA control ($P < 0.05$). $^{\#}$Different from corresponding obese ($P < 0.05$).

Figure 3.3. Respiratory capacity in human primary myotubes from lean and obese individuals in response to substrate challenges.



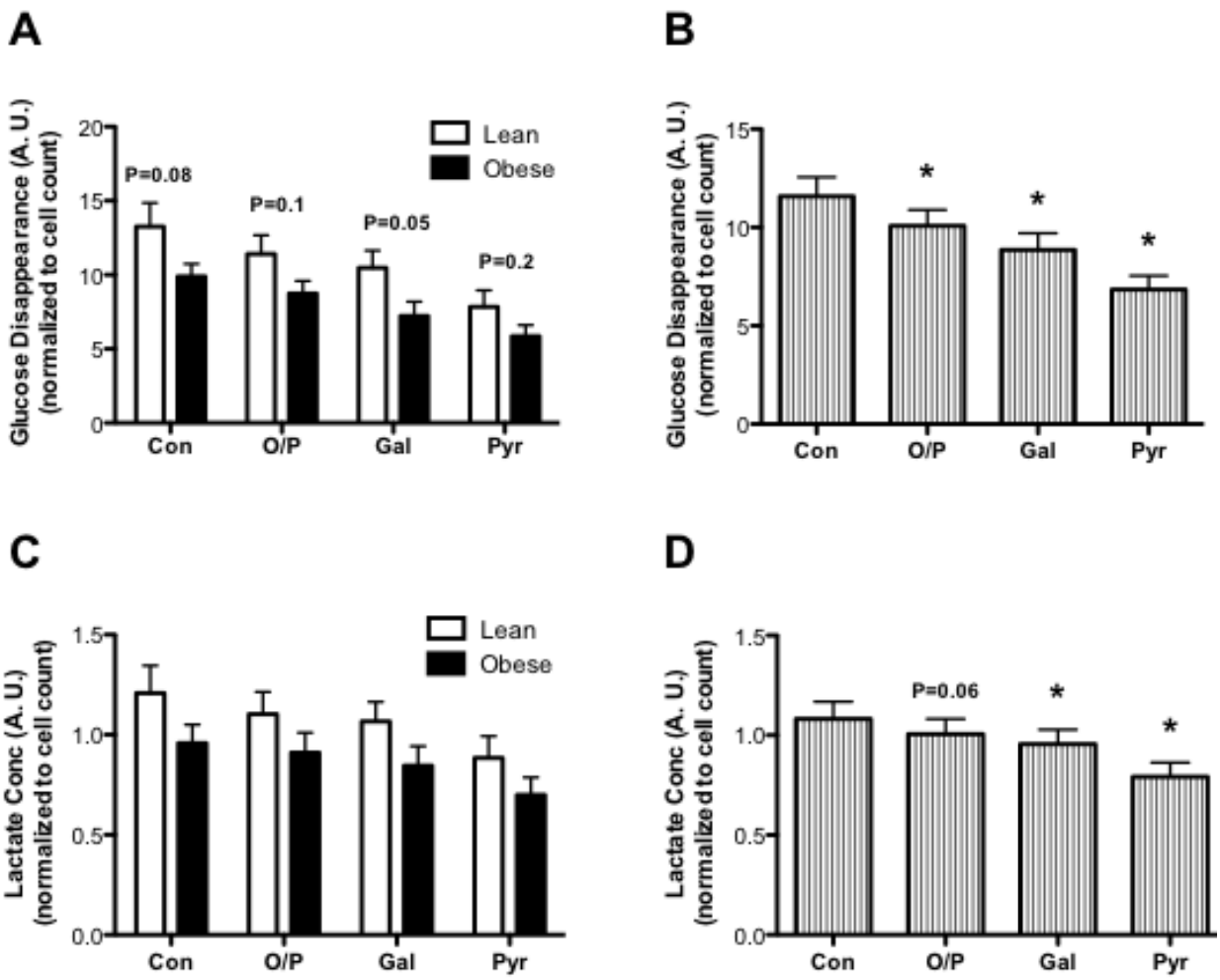
Fully differentiated myotubes were incubated for 24 hours in the presence of either 100 μM oleate/palmitate (O/P) **(A)**, 10 mM galactose (Gal) **(B)**, or 10 mM pyruvate (Pyr) **(C)**, all of which were added directly to the differentiation media. Following this 24-hour incubation, myotubes were harvested, permeabilized and oxygen consumption was assessed (**A-D**). Rates of O_2 consumption in response to PC – palmitoyl carnitine [25μM]; M - malate [2mM]; ADP [4mM] *(PCM$_3$)* Cyto C – cytochrome C [10μM]; G – glutamate [10mM]; S – succinate [10mM]; FCCP - Carbonyl cyanide p trifluoro methoxyphenylhydrazone [2μM]. (**D**) Data from lean and obese subjects were pooled to illustrate the effects of each substrate tested. Data represent mean ± SEM; n = 9-10 (**A-C**), n = 19 (**D**). *Main effect of treatment ($P < 0.01$). There was no effect of group. #Different from corresponding control condition ($P < 0.01$).

Figure 3.4. Basal and FCCP-stimulated respiration within intact primary human myotubes.



Basal and FCCP [5μM]-stimulated respiration was assessed in intact primary human myotubes, following a 24-hour incubation with 100 μM oleate/palmitate (O/P), 10 mM galactose (Gal), or 10 mM pyruvate (Pyr) (**A-B**). Assays were performed in differentiation media alone (Con) or in differentiation media identical to the media the cells were originally incubated in for 24 h (O/P, Gal, or Pyr) **(A)** Pooled data from lean and obese subjects. **(B)** Lean and obese responses. Data represent mean ± SEM; n = 14-16 (**A**), n = 7-8 (**B**). *Different from corresponding basal control condition ($P < 0.05$). $^{\#}$Different from corresponding control-FCCP condition ($P < 0.05$).

Figure 3.5. Glucose disappearance and media lactate concentration in response to 24 h substrate challenges in human primary myotubes from lean and obese individuals.



Fully differentiated myotubes were incubated for 24 hours in the presence of either 100 μM oleate/palmitate (O/P), 10 mM galactose (Gal), or 10 mM pyruvate (Pyr), all of which were added directly to the differentiation media. (**A**) Glucose disappearance during the 24-hour incubation period in lean and obese. **(B)** Pooled data of glucose disappearance from lean and obese subjects. (**C**) Lactate concentration of the media after the 24-hour incubation period in lean and obese. (**D)** Pooled data of media lactate concentration from lean and obese subjects. Data represent mean ± SEM; n = 7-8 (**A, C**), n = 14-16 (**B, D**). *Different from corresponding control condition ($P < 0.05$).

CHAPTER 4: INTEGRATED DISCUSSION

In the past 40 years the prevalence of adult obesity in the United States has increased dramatically from 11.9% to 35.5% in men and from 16.6% to 35.8% in women (53, 54). Coinciding with the increased prevalence of obesity were the findings that an elevated whole body respiratory quotient (RQ) was predictive of future weight gain (211) and that skeletal muscle RQ was elevated in obese individuals (94). The increased reliance on carbohydrate (CHO) in obese individuals has led some investigators to question whether the increase in CHO oxidation was, in part, compensation for an inability to oxidize fatty acids (82). Twenty-five years of investigation of fatty acid metabolism by our laboratory has revealed depressed fatty acid oxidation in the skeletal muscle of obese individuals utilizing numerous methodologies: intact muscle strips (88), muscle homogenates from vastus lateralis biopsies (97), *in vivo* tracers (183), and in human primary skeletal muscle cell culture (11, 36, 87). From these studies we have hypothesized that the inability to oxidize fatty acids in obese individuals may have contributed to their positive lipid balance and weight gain (52, 149). Numerous investigators have shown that weight loss drastically improves metabolic health but has little effect on the ability of previously obese individuals to oxidize fatty acids (5, 14, 94, 159, 183), leaving them prone to weight regain (5, 55, 118, 119, 191). Our laboratory has also shown that 10 consecutive days of aerobic training rescues fatty acid oxidation (FAO) by increasing skeletal muscle FAO in lean, obese, and previously obese individuals to the same extent (14), making exercise one of the few interventions to correct the impaired ability to oxidize fatty acids in the obese individual. Furthermore, our lab (10, 22, 23) and others (5, 182) have found that obese individuals demonstrate an impaired response to upregulate FAO in the face of a lipid challenge. The

inability to upregulate FAO in response to increased dietary fat may result in a positive lipid balance and leave the obese individual vulnerable to future weight gain.

The current investigation had two primary aims. Aim 1 was to develop an *in vitro* model of short-term exercise training (contractile activity) to determine the mechanisms responsible for normalizing FAO in obese and previously obese individuals. The second aim of this investigation was to determine whether the inability of the skeletal muscle cells from obese individuals to respond to increases in dietary lipid was unique to dietary lipid or whether the inability to switch between substrates encompassed other oxidizable substrates as well.

Adaptations to Short-Term Exercise Training *In Vivo*

Aerobic training increases the reliance on fatty acids during exercise (78, 120) and in some cases at rest (159). Cross sectional studies have demonstrated that aerobically fit individuals possess a high skeletal muscle oxidative capacity and increased rates of fatty acid oxidation during submaximal exercise (100, 186, 193). The evidence from cross sectional and aerobic training studies have lead to the conclusion that enhancing skeletal muscle oxidative capacity will increase the reliance on fatty acids. However, the increase in FAO during short-term exercise training (one bout to ~10 d) does not always coincide with increased oxidative capacity, thus disconnecting this relationship.

Untrained individuals are highly reliant on carbohydrate oxidation during exercise training. For example, in an untrained individual, a single acute bout of aerobic exercise for 60 min at 62% VO_2max decreases muscle glycogen content by 73.2% and increases muscle lactate by 7.1 fold (73). By comparison, four weeks of aerobic training in these same individuals resulted in muscle glycogen content reductions of only 20.4% and lactate increases of only 2.8 fold upon the completion of 60 min of exercise at the same absolute intensity (73). One of the

earliest adaptations to aerobic exercise training is a decreased reliance on CHO and a decrease in lactate production; as few as three consecutive days of exercise training has been shown to dramatically decrease muscle lactate accumulation (70). Although data for the ability of three consecutive days of aerobic exercise to increase FAO is lacking, FAO has been shown to increase in muscle homogenates from the vastus lateralis of previously untrained individuals with as little as seven days of aerobic training (10).

A caveat to the switch in substrate utilization from predominately CHO to increased fatty acid utilization during short-term aerobic exercise training is there is often no change in skeletal muscle oxidative capacity during this training period, thus disconnecting the relationship between skeletal muscle oxidative capacity and substrate utilization. Short-term training studies consisting of 3 d (70), 5 d (141), 5-7 d (72), and 10-12 d (74) of consecutive training have shown that the activities of succinate dehydrogenase (SDH), citrate synthase (CS), and β-hydroxy-acetyl coenzyme A dehydrogenase (β-HAD) have not yet increased whereas longer training durations of 14 d (176) and 31 d (141) robustly increase these same markers of mitochondrial content in skeletal muscle. It should be noted that some short-term training studies of 6-10 d have increased skeletal muscle mitochondrial content (32, 169), however this is not always the case. In a recent study in our own laboratory, seven consecutive days of aerobic training increased FAO in muscle homogenates and CS but not OXPHOS and COX IV protein or β-HAD activity (10).

It appears that seven consecutive days of aerobic training may be at or around the crossroads where the reliance on fatty acids increases before changes in mitochondrial content comprehensively occur. The goal of the present investigation was to establish an *in vitro* contractile activity model in human primary skeletal muscle cells capable of mimicking some of

the adaptations to short-term exercise training such as increasing the reliance on FAO and at the same time increasing markers of mitochondrial content and capacity. To achieve these objectives, two types of electrical stimulation protocols, *intermittent* (3 h/d 2-4 d) and *continuous* (48 consecutive h), were employed in this investigation.

Intermittent (3 h/d 2-4 d) Electrical Stimulation

The main finding of the *intermittent* electrical stimulation investigation was that short-term (3 h/d, 2-4 d) electrical stimulation of human primary skeletal muscle cells (HSkMC) was ineffective in mimicking the *in vivo* effect of short-term exercise training due to the model's inability to consistently increase FAO (Figures 2.1A-D) or markers of mitochondrial content (Figure 2.2A). Although the small sample sizes from individual experiments prevent definite conclusions from being drawn, this investigation utilized more than five electrical stimulation protocols (28, 42, 131, 145, 188) and well over 100 trials in HSkMC and C2C12 cells and was unable to demonstrate a consistent increase in either mitochondrial content or FAO. The videos that we (Supplementary Materials; Videos 2.1-2.4) and others (107, 117, 121, 129, 131, 148) have produced demonstrate the excitability of cultured cells under electrical stimulation pacing. There are also a number of investigators who have demonstrated that acute stimulation results in calcium cycling (48, 101, 103, 117, 190) as well as increases in the mRNA of transcriptional (28, 131, 165, 188), and oxidative genes (28, 131, 188). However, to date, Hood and colleagues are the sole investigators to observe functional changes in transcription factor and oxidative protein, or oxygen consumption in response to intermittent electrical stimulation (89, 188). In their first investigation Irrcher et al (89) observed an increase in transcription factor and coactivator protein (TFAM, PGC-1α, and NRF-1) but not mitochondrial protein (Cty C) after 4 days of intermittent electrical stimulation in C2C12 cells. From these data it is tempting to speculate the duration of

electrical stimulation was great enough to increase transcription factor protein, yet, insufficient to translate into functional oxidative protein. However, in a later investigation using a similar but less intense 4-day intermittent protocol (9 V vs. 55 V), Uguccioni & Hood (188) observed increased TFAM and COX IV protein as well as COX activity and intact cell respiration as measured by a Clarke electrode, indicating mitochondrial protein content was enhanced. In the current investigation, the electrical stimulation parameters utilized by Hood and colleagues (89, 188) resulted in either no increase in COX IV protein (C2C12, HSkMC) (Figures 2.2A, 2.3A, & 2.4A) or cell death (HSkMC). Although the pulse generator and stimulator plates used in the previous studies and current investigation are different the exact cause of the experimental differences remains unclear.

One factor that may have prevented the intermittent electrical stimulation protocols from facilitating any appreciate changes in our desired metabolic outcomes in HSkMC is their relative lack of excitability. The excitability of cultured cells is greatest in rat and mouse primary cells and C2C12 cells possess "exceptionally low excitability" (41). Our observations have shown the excitability of HSkMC to be several orders of magnitude less excitable than C2C12 cells (Supplementary Materials; Videos 2.1-2.4). We felt the general lack of excitability in HSkMC combined with our desire to observe a training effect may have necessitated longer periods (48 h) of electrical stimulation and a greater number of myotube contractions before changes in oxidative capacity were to occur.

Continuous (48 h) Electrical Stimulation

In the present study, continuous electrical stimulation for 48 h was found to resemble a mixture of adaptations similar to both a) short-term exercise training and b) a single acute prolonged exercise bout. In short-term *in vivo* exercise training, daily, consecutive training bouts

result in transient adaptations, the accumulation of which increases oxidative capacity over the course of several weeks. In turn, there is a decreased reliance on CHO, glycogen sparing, decreased lactate accumulation, and increased FAO during each successive training bout (70, 73, 176). The adaptations in substrate selection, namely decreased lactate accumulation and increased FAO, are believed to be the direct result of an increased oxidative capacity. On the other hand, an increase in FAO during a single acute prolonged exercise bout is primarily the result of glycogen depletion, not an increase in oxidative capacity.

In the present investigation, 48 h of electrical stimulation *in vitro* did not increase OXPHOS protein or respiratory capacity (Figures 2.6C & D). These observations suggest that 48 h of electrical stimulation in culture is not comparable to the very minimum amount (6-10 d) of *in vivo* endurance training necessary to increase skeletal muscle oxidative capacity and is probably more similar to a single acute bout of prolonged exercise, which would not be expected to increase oxidative capacity.

Another very early adaptation in response to short-term *in vivo* training is a reduction in muscle and blood lactate accumulation in subsequent training bouts (70). In the present investigation, media lactate concentration increased at 12 h of electrical stimulation and remained elevated throughout the 48 h stimulation period despite fresh media changes every 12 h (Figure 2.6A). The failure of media lactate not to decrease during the latter stages of the 48 h stimulation period suggests the myotubes remained reliant on glycolysis, and although increased media lactate is indicative of a stimulation effect, the pattern of lactate accumulation in the media is more indicative of a single acute bout of exercise than it is of the cumulate effects of short-term exercise training.

In the present investigation, forty-eight hours of continuous stimulation was able to increase FAO (Figure 2.7A). Although it is difficult to ascertain whether the increase in FAO was more similar to a single prolonged bout of contractile activity or short-term exercise training, there is evidence for both. Media glucose did not differ between control and stimulated cultures, yet lactate was elevated in the stimulated group throughout the 48 h period (Figures 2.6A & B). An attractive explanation for these findings would be that the continuous nature of the 48 h stimulation bout is similar to a single prolonged training bout in that they both promote the breakdown of glycogen. In turn, increased glycogenolysis would contribute to the elevated media lactate and as a result of reductions in glycogen content, FAO increased to meet energy demand. Evidence supporting the hypothesis that, in culture, glucose is preferentially utilized at the expense of FAO comes from several electrical stimulation investigations that have demonstrated glucose uptake or oxidation increases after 30 min (145), 1 h (117), 8 h (107), 24 h (131), and 48 h (131). On the other hand, in the investigations containing prolonged continuous stimulation bouts, FAO did not increase after 24 h (107, 131) but did so after 48 h (131). The observation that FAO did not increase after 24 h but did so after 48 h may be similar to *in vivo* exercise data, in which the initial increase in FAO during the early stages of exercise is relatively small compared to the substantial increase in FAO during the later stages of an extended prolonged exercise bout. For example, Bradley et al (24) demonstrated that when recreationally active individuals cycled for 120 min at 60% VO_2 peak, FAO remains relatively constant from the initial stages of exercise through 80 min before increasing between 80 and 120 min (24). The increased reliance on FAO during the later stages of exercise likely coincides with a depletion in glycogen content. Taken together, the data from this investigation seems to indicate that *in vitro*

contractile activity is capable of increasing glucose utilization, and if performed over an extended period of time (48 h), may increase FAO as well.

Alternatively, an early response to short-term aerobic training (1-3 d) *in vivo* is an increase in the monocarboxylate transporters (MCT) (70, 71), which are responsible for exchanging pyruvate for lactate across the sarcolemma. In the present investigation, elevated media lactate may have simply been the result of an increased exchange of media pyruvate for myocyte lactate due to elevated myocyte MTC expression, rather than increased glycogenolysis. Although not measured here, if true, increased MCT expression after a single 48 h bout of stimulation would support this model as being similar to short-term exercise training instead of simply a single extended exercise bout.

The increase in FAO in the present investigation may have been an early adaptation in fuel selection in response to the continuous 48 h short-term training bout, as demonstrated in previous short-term (7-10 d) *in vivo* exercise training studies (10, 14). In the present investigation, FAO was measured approximately 15 hours after the termination of the 48 h stimulation bout and although glycogen content was not measured, it is plausible that glycogen content was replenished during this time; implicating a mechanism other than glycogen depletion to be responsible for the observed increase in FAO. Our laboratory has demonstrated FAO increases in response to seven consecutive days of aerobic training (10), however, to date no investigations have looked into the effects of short-term aerobic training of less than seven days on FAO, highlighting the need for future research during this time frame.

Taken together, it appears that 48 h of continuous electrical stimulation may be of a sufficient duration to increase FAO, but not great enough to illicit increased OXPHOS protein content, respiratory capacity, or decreased lactate accumulation. In those regards, 48 h of

continuous electrical stimulation recapitulates many of the effects of a single acute bout of prolonged aerobic exercise but is unable to mimic many of the effects of short-term aerobic training. For the complete effects of short-term training including increased skeletal muscle oxidative capacity to be observed, future electrical stimulation investigations in HSkMC should extend the stimulation period beyond 48 h. Doing so, may elucidate the timeline and mechanisms behind an increase in FAO due to short-term aerobic training before and during the time adaptations in oxidative machinery occur.

The Effect of the Addition of Oxidizable Substrates on Metabolic Flexibility

The second aim of this investigation was to determine whether HSkMC from severely obese donors were similarly *metabolically inflexible* in response to a CHO (galactose or pyruvate) overload as they are to a lipid challenge. The primary finding of this investigation was that, in culture, the skeletal muscle of the severely obese individual is *metabolically flexible* as it retains the ability to upregulate OXPHOS protein (Figures 3.2A-B), CS (Figures 3.2A-B), and oxidative capacity (Figures 3.3A-C) in response to not only galactose and pyruvate, but lipid as well. These findings are in contrast to previous data generated in our laboratory demonstrating that lean, but not obese, increase the capacity of state 3, palmitoyl-carnitine malate supported respiration (PCM_3) (23) in response to a lipid incubation. Differences in methodology and inherent human subject variability are likely contributors to these differences. The results of the current investigation support the hypothesis that *metabolic flexibility* is an inherent trait of skeletal muscle and that extramuscular mechanisms may be responsible for the metabolic inflexibility observed *in vivo* (61). It is therefore imperative to compare and contrast the *in vitro* and *in vivo* experimental results to gain a better understanding of the factors controlling metabolic flexibility.

Metabolic flexibility is broadly defined as the ability to match nutrient oxidation with nutrient availability. The two most common means of testing metabolic flexibility *in vivo* are a) a hyperinsulinemic clamp and b) the imposition of a high fat diet (HFD). In the late 1990's David Kelley's group found that, compared to their lean counterparts, obese individuals rely more heavily on glucose oxidation during times of fasting and less on glucose oxidation during insulin stimulation (94, 96). Galgani et al (57) has attributed the metabolic inflexibility in obese and diabetic individuals described by Kelley et al (94) primarily to insulin resistance, as normal metabolic flexibility is exhibited when metabolic flexibility is normalized to glucose uptake. The design (no insulin) and results of the current investigation are more similar to the HFD literature and as such will be discussed in the context of previous HFD studies.

With the imposition of a HFD obese individuals are thought to match nutrient oxidation with substrate availability (upregulate FAO) more slowly than their lean counterparts (182). The delay in time between the increase in dietary fat and the time required to match nutrient oxidation with substrate availability may cause the obese individual to store more and oxidize less fat resulting in a positive fat balance (167, 182). Numerous, repetitive bouts of these "mismatches" are thought to lead to increased adiposity and weight gain. On the other hand, lean individuals adapt to the HFD more rapidly thereby minimizing the opportunity for positive fat balances to occur.

There are, however, several instances where the lean individual does not respond to increases in dietary fat. For example, in young, lean individuals, Schutz et al (162) supplemented a normal mixed diet for 24 h with 987 kcal of fat (52% of total energy) and noted no change in the subsequent 36 h RQ indicating no increase in FAO. When CHO continues to be present, in adequate quantities, substrate utilization will not change from CHO to fatty acids

regardless of the amount of dietary fat. In an overfeeding study, 40 nonobese men and women were overfed ~1040 kcal for 3 d. RQ actually increased, from 0.81 to 0.85 despite 46% of kcal provided derived from fat (156). When CHO intake is controlled, as in a isocaloric HFD, it may take up to 7 d for lean individuals to completely upregulate FAO to match fat intake (161). When exercise is superimposed upon a HFD in lean individuals, a lower steady state RQ is reached at 3 d compared to the HFD alone (166). This seems to indicate that physical activity is an important factor in determining how quickly an individual responds to the HFD.

Schrauwen et al (160) has demonstrated that obese individuals are capable of adapting to a HFD within 36 h when a glycogen exhausting exercise bout (~60 min) is performed prior to the initiation of a HFD. From the results of this study, Schrauwen et al (160) concludes "the results of the present study demonstrate that obese subjects are capable of rapidly adjusting fat oxidation to fat intake when glycogen stores are lowered. Therefore, these results are in concordance with the results obtained in lean subjects and do not provide evidence for an impaired capacity to rapidly change fat oxidation in obese subjects."

In addition, not all HFD studies have demonstrated a blunted ability for obese individuals to upregulate FAO in response to increased dietary lipid alone. In a group of moderately obese individuals (BMI 35.9 ± 4.9), Bergouignan et al (17) indicated that switching from a 20% to 50% HFD for 2 d decreased 24 h whole body RQ and increased markers of skeletal muscle oxidative capacity in lean and obese individuals to a similar extent. There were also no differences in CHO or fat balances noted between the groups, indicating the potential for weight gain between groups was equivalent. A strength of the Bergouignan et al (17) study is that it was conducted in a metabolic chamber. Therefore, a subject's activity level and daily caloric expenditure can be

carefully monitored and controlled. Free-living experiments afford no such control allowing the opportunity for unintentional differences in energy expenditure to occur (17, 92, 110, 111).

From the studies of Bergouignan et al (17) and Schrauwen et al (160) it would appear that when diet and physical activity are meticulously controlled, the ability of skeletal muscle from lean and obese individuals to respond similarly to oxidizable substrates should not be unexpected. In the cell culture model when glucose concentrations, insulin, and fatty acid concentrations are identical between the lean and the obese, metabolic flexibility remains intact. The findings in the current investigation support the notion that regulation, not oxidative capacity, dictates metabolic flexibility (57, 61). It should be pointed out however, that although not statistically significant, the obese groups in the present study and the Gaster et al (61) investigation demonstrated decreased responsiveness to metabolic stimuli. It is possible that increasing the concentration of lipid may have shown a lean-obese effect. How this contributes to obesity will continue to be a debate.

The Role of Energy Balance and Carbohydrate in the Development of Obesity

The effect of fatty acid oxidation on weight gain and weight loss remains controversial. There are as many published reports indicating that a high RQ is associated with or predictive of future weight gain (25, 43, 47, 49, 118, 119, 153, 164, 191, 211) as there are that report no association (93, 113, 163, 179, 197-199, 202, 203). The investigations linking elevated RQ values with future weight gain, although significant, tend to explain less than 30% of the variance (49, 119, 164, 211) in weight gain between individuals. On the other hand, Weinsier et al (197) followed two groups of women over the course of 1 yr to determine characteristics associated with the propensity to regain weight and found that those who regained a substantial

portion of weight lost (>10%) had significantly reduced rates of free-living activity energy expenditure which explained an astonishing 77% of their weight regain.

The evidence for reduced FAO in the presence of obesity is also unclear. For example, our laboratory has demonstrated that moderately obese (BMI, 30.2 ± 0.8 $kg \cdot m^2$) individuals do not have diminished rates of FAO whereas severely obese (BMI, 53.8 ± 0.4 $kg \cdot m^2$) individuals possess much lower rates (88) suggesting that in severe, but not moderate obesity, FAO is diminished. On the other hand, whole body FAO determined by RQ have demonstrated that subjects with BMI values of 49 ± 2 kg/m^2 (124) and 56.3 ± 2.7 kg/m^2 (137) have very high rates of FAO. Blaak et al (20) stratified a group of 701 obese individuals into BMI categories of 30-35, 35-40 and >40 kg/m^2 and found that as BMI increased, so did fasting FAO whether unadjusted, adjusted for fat free mass or expressed as a percentage of resting energy expenditure. Human primary skeletal muscle cell culture (HSkMC) has also yielded conflicting results as some studies (11, 36, 37, 62, 87) but not others (31, 61, 171) have shown decreased rates of FAO. Taken together, it is difficult to ascertain whether FAO is reduced in the obese state and if decrements in FAO are present, whether this is a cause or a consequence of obesity.

High fat diets are a commonly used tool in the study of obesity and its relationship to metabolic flexibility. Although HFD's are a useful experimental model, the results of such studies should be interpreted with caution, as they do not accurately reflect the dietary habits of the public. In the past 40 years the prevalence of adult obesity in the United States has increased dramatically from 11.9% to 35.5% in men and from 16.6% to 35.8% in women (53, 54). According to National Health and Nutrition Examination Survey (NHANES) data, during that same time CHO intake has increased from 44.0% to 48.7% and fat intake has actually decreased from 36.6% to 33.7% (7). There are also no reported differences in the macronutrient intake

between lean, overweight, and obese individuals, however, each group has significantly increased total caloric intake over the previous 40 years (7). The efficacy of utilizing a multiple day high fat diet containing greater than 50% does not accurately reflect dietary habits in the United States. For example, outside of salad dressing and condiments the fast food restaurant, McDonalds, does not contain a single item containing > 59% fat. People typically consume sugary beverages and French fries as part of their meal, further decreasing the contribution of dietary fat from the meal as a whole.

It has been suggested that severely obese and diabetic individuals possess an inherent defect in skeletal muscle that prevents them from oxidizing fatty acids to the same extent as lean individuals. Perhaps a new paradigm would suggest that obese individuals do not oxidize fatty acids because they are not required to do so. There is overwhelming evidence that macronutrient oxidation is governed by an "oxidative hierarchy". When CHO is present in the diet, CHO is preferentially oxidized at the expense of FAO. Obesity is the result of a prolonged energy imbalance between energy intake and expenditure. Diets high in fat tend to also contain a greater number of calories due to the caloric density of fat. For example, when subjects were asked to eat *ad libitum* from diets containing low-fat, medium-fat, and high-fat without knowledge of the macronutrient composition, energy intakes significantly increased with percent fat (173). Continual access to food and opportunities to eat ensures that CHO stores are never diminished to the point where an increased reliance on fatty acids is necessary. Although the percentage of fat in the diet decreased during the past 40 yrs, the total amount of fat in grams remained the same (7). During this same time, the proportion of calories coming from CHO has increased from 44.0% to 48.7%, giving the body no other choice than to use CHO and not oxidize fat.

Conclusion

Due to the environment we live in, it is difficult to not become overweight or obese in the United States (53, 140). During the past 40 yrs the prevalence of obesity has tripled in the United State while the consumption of dietary fat has actually decreased (7). In epidemiological studies, countries with the highest per capita fat intake oftentimes do not have the most obese population (113). Yet, we are still focusing our efforts to reduce body weight through targeting dietary fat and fatty acid oxidation. The real culprit in the obesity crisis is not fat it is carbohydrate. Although probably oversimplistic, Flatt's two- compartment model forms the lens though which obesity should be viewed (51). The body's initial defense against changes in body weight homeostasis is glycogen content and/or a high carbohydrate availability. When glycogen stores are plentiful, CHO is the preferred fuel choice, when glycogen stores are depleted, fat becomes the preferred fuel of choice. It is not until glycogen content and carbohydrate availability is lowered, through fasting, diet and/or exercise that fat stores can begin to be depleted. High fat diets are used by individuals to lose weight (60, 86, 155) but are also blamed for individuals to gain weight (25). This highlights the importance of energetic balance, not macronutrient composition. Metabolic flexibility may (5) or may not (17, 122) cause fat imbalances in the short term, however the results of these studies are inconsistent with and not reflective of the average individual's dietary habits. HFD studies often use in excess of 60% of calories from dietary fat (3, 10, 19, 22). A concerted effort is required to achieve $\geq$ 50% of calories from fat in the typical diet; even Atkins and South Beach dieters have trouble consistently achieving this mark (60, 116). It is indisputable that aerobic exercise increases oxidative capacity and confers numerous health benefits. The ability of exercise to change resting substrate utilization is less clear. Exercise should be used as a tool to expend energy and

deplete glycogen stores, which may aid in achieving weight loss. Regardless of whether you exercise a lot or not at all, eat a diet consisting of a high percentage of fat or very little, energetic balance is the key to weight loss and weight gain.

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APPENDIX: IRB APPOVAL

EAST CAROLINA UNIVERSITY
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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: 12/13/2012
Re: CR00000684
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 12/12/2012 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 12/12/2012 to 12/11/2013.

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
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:

R. Hickner