ABSTRACT

Metabolic Inflexibility in Skeletal Muscle with Obesity

by

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The skeletal muscle of obese individuals has a reduced capacity to oxidize lipids. The hypothesis to be tested in this dissertation is that the ability to regulate lipid oxidation in response to lipid exposure is impaired in skeletal muscle of obese individuals. An inability to appropriately respond to metabolic stimuli has been termed "metabolic inflexibility" and has been linked with obesity and insulin resistance. To test this hypothesis, two models of lipid exposure were utilized: a 5 day high fat diet (HFD) and lipid incubation in primary myotubes cultured from lean and obese donors. Trend analyses indicated that mRNA content of genes linked with fat oxidation were collectively up-regulated with the HFD in skeletal muscle of lean but not obese subjects, suggesting a global response that is indicative of skeletal muscle mitochondrial dysfunction in obesity. Specifically, there was a 2-fold increase ($P < 0.05$) in fasted PDK4 content following the HFD in leans, while the obese participants tended to have

decreased PDK4 content; and UCP3 mRNA content decreased by almost half in the obese, but not lean participants. In the second series of studies, a 24 h lipid incubation increased mitochondrial respiration by up to 2-fold in the presence of lipid and carbohydrate in myotubes from lean donors in both State 3 and uncoupled respiration ($P < 0.05$), though there was no change in cells cultured from the obese donors. In addition, mitochondrial DNA content increased by 16% ($P < 0.05$) with lipid exposure in cells from lean subjects but tended to decrease in myotubes from obese subjects (13%; $P = 0.06$). The presence of these defects in culture suggests a genetic or epigenetic origin with obesity. Together, these data support the hypothesis that the skeletal muscle of obese individuals is metabolically inflexible and provides the novel information that this inflexibility extends to the ability to respond to lipid exposure in human skeletal muscle.

METABOLIC INFLEXIBILITY IN SKELETAL MUSCLE WITH OBESITY

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by

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ADP: adenosine diphosphate

ATP: adenosine triphosphate

AU: arbitrary units

BCA: bicinchoninic acid

BMI: body mass index (kg/m^2)

BSA: bovine serum albumin

β-NADH: nicotinamide adenine dinucleotide

β-HAD: β-hydroxyacyl coenzyme A dehydrogenase

CPT1: carnitine palmitoyl transferase 1

CO_2: carbon dioxide

CoA: coenzyme A

COX-IV: cytochrome C oxidase, isoform IV

Ct: cycles to threshold

DNA: deoxyribonucleic acid

DM: human skeletal muscle differentiation media

DMEM: Dubelco's Modified Eagle's Serum

DMSO: dimethyl sulfoxide

DTNB: 6,6'–dinitro–3,3'–dithiodibenzoic acid

EDTA: ethylenediaminetetraacetic acid

EGTA: ethylene-bis(oxyethylenenitrilo)tetraacetic acid

FAO: fatty acid oxidation

FBS: fetal bovine serum

GM: human skeletal muscle growth media

H_2O_2: hydrogen peroxide

HEPES: 4- (2-hydroethyl)-1-piperazineethenesulfonic acid

HFDL high fat diet

HOMA: homeostasis model assessment

HSMC: human skeletal muscle cell

IMCL: intramyocellular lipid

IMTG: intramyocellular triglyceride

JO_2: oxygen flux (pmol O_2*(s*2 mL)$^{-1}$)

Lean: BMI < 25 kg/m^2

MCAD: medium chain acyl-CoA dehydrogenase

Morbid Obesity: BMI > 40 kg/m^2; also referred to as extreme obesity

mRNA: messenger ribonucleic acid

NEFA: non-esterified fatty acid

O_2: Oxygen

Obesity: BMI > 29.9 kg/m^2

Overweight: BMI < 24.9 kg/m^2, BMI > 30 kg/m^2

PCM: palmitoyl carnitine and malate

PCR: polymerase chain reaction

PDH: pyruvate dehydrogenase

PDK4: pyruvate dehydrogenase kinase, isoform 4

PGC-1α: peroxisome proliferator-activated receptor gamma coactivator 1 alpha

PM: pyruvate and malate

PPARα: peroxisome proliferator-activated receptor alpha

PPARδ: peroxisome proliferator-activated receptor delta (also known as PPARβ)

PPARγ: peroxisome proliferator-activated receptor gamma

PVDF: polyvinylidine fluoride

ROS: reactive oxygen species

RTQ-PCR: real-time quantitative polymerase chain reaction

SDS: sodium dodecyl sulfate

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: standard error of the mean

UCP3: uncoupling protein, isoform 3

VO_{2max}: maximal oxygen consumption (mL O_2/min)

CHAPTER 1: REVIEW OF LITERATURE

INTRODUCTION

Obesity is a public health concern that creates a large strain on our health care system. Skeletal muscle from obese individuals appears to have a lower capacity for lipid oxidation than their lean counterparts (Hulver et al., 2003), which may provide a potential mechanism for metabolic inflexibility, weight gain, and insulin resistance with obesity. Interestingly, differentiated, cultured skeletal muscle cells (myotubes) from extremely obese individuals retain this defect in oxidation (Hulver et al., 2005), thus offering an intriguing and suitable model for hypothesis testing that cannot be performed *in vivo*. The over-arching hypothesis to be tested in the work performed for this dissertation was that the defect in the ability to utilize lipid in the skeletal muscle of extremely obese individuals is linked with impairments in mitochondrial function, specifically the ability to upregulate factors linked with lipid oxidation in response to lipid exposure. We tested this hypothesis by examining: 1) the responses of genes linked with lipid oxidation to a high fat diet in lean and obese individuals and 2) the response to lipid exposure in myotubes derived from lean and obese donors.

It has been demonstrated that lean and obese individuals respond differently to a metabolic challenge, such that lean humans increase carbohydrate oxidation in response to insulin stimulation whereas obese humans do not (Kelley et al., 1999). In addition, a short-term high fat diet fails to upregulate lipid oxidation, as is observed in

lean individuals, in previously obese men and women (Astrup et al., 1994). This inability to respond to substrate challenge may be an integral component of the metabolic anomalies present with obesity. We hypothesized that a lipid challenge would increase the mRNA content of genes in skeletal muscle involved with lipid oxidation in lean individuals, but that obese obese subjects would not demonstrate a similar upregulation. To further examine the skeletal muscle response to a substrate challenge in obesity, we measured mitochondrial respiration in the human cell culture model (primary skeletal muscle myotubes). We hypothesized that permeabilized myotubes from obese and extremely obese individuals would exhibit lower respiration rates than cells from leans with lipid as the substrate. Specifically, whereas cells from lean humans were hypothesized to have increased respiration as a result of lipid exposure (i.e. metabolic flexibility), cells from obese donors would not respond to the lipid with a similar increase in respiration, indicating that the mitochondrial aberrations observed in human skeletal muscle tissue are preserved in this cell culture model.

THE OBESITY EPIDEMIC

Obesity in the Unites States is a growing epidemic, with rates reaching 34% in 2006 (Centers for Disease Control, Prevalence of Obesity in the United States, NHANES 2003-2004). The incidence of obesity has doubled in adults and tripled in children and adolescents from 1980 to 2004 (Ogden et al., 2006). Inherent with this elevation in prevalence is a concomitant rise in obesity-related health concerns that comprise the

metabolic syndrome, such as hypertension (Brown et al., 2000), hyperlipidemia (Gregg et al., 2004), and diabetes (Gregg et al., 2004; Gregg et al., 2005). Obesity-related health concerns are responsible for approximately $140 billion annually in direct and indirect medical costs and some estimate that, each year, approximately 400,000 deaths are attributable to obesity and obesity-related diseases (Finkelstein et al., 2005). Given these trends, obesity will only continue to place greater financial strain on all branches of the healthcare system. While the financial and social consequences of the obesity epidemic are daunting, little is known about the cellular processes that contribute to obesity and the mechanism by which further complications arise (i.e. insulin resistance).

Body weight, BMI, and abdominal adiposity have all been correlated with insulin action (Goodpaster et al., 1997) and rising rates of obesity are inextricably linked with rising rates of type 2 diabetes. It is estimated that 40% of the adult population in the Unites States is hyperglycemic (> 100 mg/dL fasting glucose levels), while only 13% of the population has been diagnosed with diabetes (NHANES 2005-2006 (Cowie et al., 2009)). In 2004, 83% of type 2 diabetes diagnoses were made in those with BMIs greater than 35 kg/m^2; and the rate of diabetes in this population has more than doubled in the past 20 years (Gregg et al., 2004). The exact relationships between these two disease states (obesity and type 2 diabetes) remains elusive, although these groups remain phenotypically similar in that both are characterized by increased body fat

percentage, decreased ability to clear glucose from the circulation, and impairments in oxidative metabolism (Kelley et al., 1999; Kelley and Mandarino, 1990).

Summary. Obesity is a health concern which will continue to strain our healthcare system. It is important to investigate potential cellular mechanisms responsible for the underlying causes of obesity in order to design and implement effective treatment options.

SKELETAL MUSCLE LIPID OXIDATION IN OBESITY

Skeletal muscle comprises at least 40-50% of total body mass, representing a large proportion of total body fuel metabolism and, therefore, is an important player in the metabolic anomalies associated with obesity. Studies show that intramyocellular lipids (IMCL) are more abundant in obese and type 2 diabetics than in sedentary lean individuals (Goodpaster et al., 2000) and data suggests that active lipid derivatives, such as ceramide and diacylglycerol, may be involved in the development of skeletal muscle insulin resistance (Hajduch et al., 2001; Montell et al., 2001; Shulman, 2000). The accumulation of IMCL in obesity and insulin resistance indicates that the lipid balance in skeletal muscle cells favors a net accretion, either via increased cellular uptake or production of free fatty acids and/or decreased lipid oxidation. Studies show that the ability to oxidize lipid in skeletal muscle is impaired with obesity. Kelley et al. (1999) evaluated lipid metabolism using a single leg infusion and showed lower FFA oxidation rates in obese (BMI = 34 kg/m^2) compared with lean individuals (BMI = 23 kg/m^2). The

authors also demonstrated that FFA uptake across the leg remains similar between lean and obese individuals in both the fasted and insulin-stimulated states, whereas lipid oxidation is markedly depressed with obesity (Kelley et al., 1999), suggesting that impaired lipid oxidation is a more probable factor in the etiology of obesity than elevated FFA uptake.

In more tissue-specific assessments, *rectus abdominus* homogenates from extremely obese women (BMI = 54 kg/m^2) had reduced palmitate (long chain acyl-CoA) oxidation, when compared with their lean (BMI = 24 kg/m^2) and obese (BMI = 30 kg/m^2) counterparts (58% and 83% lower, respectively) (Hulver et al., 2003). While others have observed no decrement in lipid oxidation in *rectus abdominus* strips from moderately obese individuals (BMI = 33 kg/m^2) (Steinberg, 2002), this discrepancy is likely due to the extent of obesity in which this detriment was observed. Indeed, palmitate oxidation was also impaired in *vastus lateralis* tissue of women with a greater degree of obesity (BMI = 38 kg/m^2) (Kim et al., 2000).

At the protein level, cross-sectional analyses demonstrate that obese individuals exhibit a reduction in skeletal muscle oxidative enzyme activity and content (Kelley et al., 2002; Simoneau et al., 1999). In particular the authors observed diminished activity of NADH:O_2 oxidoreductase, an index of electron transport chain capacity (Kelley et al., 2002); CPT1, which regulates entry of long chain fatty acids into the mitochondria for oxidation; and COX, an overall indicator of mitochondrial oxidative capacity (Simoneau

et al., 1999). This defect is diminished with exercise-induced weight loss (Menshikova et al., 2005) and could be responsible for reduced lipid oxidation with obesity.

Summary. Decrements in skeletal muscle lipid oxidation in obese individuals may be involved with the accumulation of excess IMCL, which has been implicated the development of skeletal muscle insulin resistance.

SKELETAL MUSCLE SUBSTRATE SELECTION IN OBESITY

Skeletal muscle lipid oxidation is normally elevated during the fasted and exercise conditions in order to preserve glucose and glycogen stores, whereas rates of lipid oxidation decline in the fed or insulin-stimulated states due to increased reliance on glucose as the metabolic substrate. This capacity for rapid changes in substrate utilization in response to availability has been termed metabolic flexibility; an inability to respond appropriately to these stimuli has been observed with obesity and type 2 diabetes (for review, see Kelley and Mandarino (2000)). In lean individuals, a high-fat diet (3-4 days, 50-55% fat) increased whole body lipid oxidation at rest (Buemann et al., 1998; Smith et al., 2000) and during exercise (Cameron-Smith et al., 2003), although obese individuals do not adapt similarly (Thomas et al., 1992). Likewise, lipid and carbohydrate oxidation is not affected by 7 weeks of a high fat diet (41% fat) in overweight individuals (BMI = 28 kg/m^2) (Landry et al., 2003). Previously obese individuals or those with a family history of obesity also exhibit a blunted increase in lipid oxidation following high fat feeding when compared with their lean counterparts

(Astrup et al., 1994; Brown et al., 2000). These studies all provide evidence that the lipid-induced metabolic flexibility observed at the whole body level may be impaired with obesity; it is not evident, however, if this metabolic inflexibility with obesity is linked with alterations in skeletal muscle.

In relation to substrate utilization in human skeletal muscle, PDK4 activity, which phosphorylates and deactivates the PDH complex to decrease glucose metabolism, is elevated in the skeletal muscle of lean individuals following 2-3 days of high fat feeding (70-73% fat) (Arkinstall et al., 2004; Pehleman et al., 2005; Peters et al., 2001). In addition, PDK4 mRNA content also increased (Arkinstall et al., 2004; Peters et al., 2001) suggesting that lipid exposure results in a rapid increase in content and/or activity of proteins regulating lipid oxidation in lean individuals. Unfortunately, similar measurements have not been made in obese individuals; thus, a primary focus of this dissertation is to determine if the inability to respond to a substrate challenge, as observed in the insulin-stimulated state (Kelley et al., 1999), carries over to skeletal muscle lipid metabolism with obesity.

Summary. Induction of skeletal muscle lipid oxidation in response to lipid exposure may be impaired in obese individuals. Although the exact cellular mechanisms remain unknown, it is possible that obese individuals do not upregulate the transcription of genes for proteins in skeletal muscle involved in lipid oxidation following lipid exposure

REGULATION OF SKELETAL MUSCLE LIPID OXIDATION

PDK4 gene expression is induced by proteins of the PPAR family. In particular, PPARα is most abundant in highly oxidative tissues such as skeletal muscle and its pharmacological activation has been shown to increase PDK4 mRNA content almost 12-fold in primary human skeletal cell culture (Muoio et al., 2002). PPARα is considered a primary mediator of lipid oxidation, specifically in the presence of lipids, such as with high fat feeding or fasting. These lipids are the endogenous ligands of PPARα and, once activated, PPARα, in conjunction with several coactivators such as PGC-1α (Vega et al., 2000), is capable of inducing gene transcription for PDK4 and UCP3, among others (Muoio et al., 2002).

The PPAR coactivator PGC-1α has also been implicated in the regulation of lipid oxidation, particularly in the presence of elevated lipid levels. In rodents, high fat feeding increased PGC-1α protein levels 2-fold (Hoeks et al., 2008). PGC-1α overexpression has also been shown to increase mRNA content of PDK4, UCP3, MCAD, and CPT1 in L6 myotubes (Koves et al., 2005).

Summary. The PPAR family of proteins, including PPARα and PGC-1α are implicated in the regulation of lipid induced lipid oxidation via induction of gene transcription of molecules directly involved with lipid metabolism.

SKELETAL MUSCLE MITOCHONDRIA AND OBESITY

The skeletal muscle of obese individuals can exhibit reduced oxidative enzyme capacity, reduced and/or smaller mitochondria, and reduced electron transport function compared with tissue from lean individuals (Kelley et al., 2002; Ritov et al., 2005; Simoneau et al., 1999). However, some would argue that these differences are largely, or entirely, based on differences in mitochondrial content.

The reduction of fatty acid metabolism in obesity and insulin resistance is often associated with accumulated intramyocellular lipids (IMCL) (Goodpaster et al., 2000), and data suggests that active lipid derivatives, such as ceramide and DAG, may be involved in the development of skeletal muscle insulin resistance (Hajduch et al., 2001; Montell et al., 2001; Shulman, 2000). Excess skeletal muscle lipid is prone to oxidation by reactive oxygen species (ROS), leading to accumulation of damaging lipid peroxides, which can reduce mitochondrial oxidative capacity in the obese and insulin resistant populations (Schrauwen, 2007). Indeed, skeletal muscle of obese men has a higher basal H_2O_2 emission than that of leans, which indicates a more oxidized cellular redox state (Anderson et al., 2009), and, as discussed previously, skeletal muscle of obese individuals presents with reduced oxidative enzyme activity and content, including NADH:O_2 oxidoreductase, CPT1, and COX (Kelley et al., 2002; Simoneau et al., 1999).

It is also well established that skeletal muscle of obese individuals has considerably lower mitochondrial content than leans when determined by

mitochondrial DNA copy number (-34% (Ritov et al., 2005)), COX protein or activity (-15-25% (Holloway et al., 2007; Simoneau et al., 1999)), or citrate synthase activity (-13% (Kim et al., 2000)). Differences in mitochondrial content may be due to differences in physical activity levels of the tissue donors, given that physical activity increases mitochondrial content (Menshikova et al., 2007) and relative inactivity of obese individuals. Many have identified reduced lipid oxidation in crude skeletal muscle homogenates of obese and insulin resistant individuals (Holloway et al., 2006; Hulver et al., 2003; Kim et al., 2000), although when normalized to total mitochondria, these differences are no longer observed (Holloway et al., 2006). Using more sophisticated measures of mitochondrial respiration, some have found this same deficit in oxidation in permeabilized muscle fibers from obese type 2 diabetics, compared with overweight control subjects, but this was no longer present when normalized to either mitochondrial DNA copy number or citrate synthase activity (Boushel et al., 2007). However, others have observed reduced oxidation in the presence of pyruvate + malate or glutamate + succinate + malate in type 2 diabetics compared with BMI-matched control subjects (Mogensen et al., 2007; Phielix et al., 2008) whether normalized to citrate synthase activity (Mogensen et al., 2007; Phielix et al., 2008) or mitochondrial DNA copy number (Phielix et al., 2008).

It remains to be seen whether the reduced lipid oxidation observed in the skeletal muscle of obese and type 2 diabetics is due to mitochondrial dysfunction per se,

or simply reduced mitochondrial content, or a combination of both. Using a novel approach of mitochondrial respiration in intact myotubes from lean and obese donors, we will investigate the role on mitochondrial content on mitochondrial respiration rates and oxidative capacity in skeletal muscle of lean and obese humans. Emerging evidence indicates that the ability to adapt to a substrate challenge, such as insulin stimulation or lipid exposure, may be an important aspect of the obese/diabetic phenotype (Astrup et al., 1994; Kelley et al., 1999). In addition, studies suggest that lipid exposure may exacerbate these phenotypic differences (Costford et al., 2008; Koves et al., 2008). In light of this we will examine oxidation rates under unstimulated and lipid stimulated conditions.

To date, most assessments of lipid oxidation in skeletal muscle have been made using radiolabelled lipids and rudimentary homogenate procedures for isolating mitochondria (Holloway et al., 2006; Hulver et al., 2003; Kim et al., 2000). The inherent flaw with these techniques is that the isolation procedure only recovers ~20% of total mitochondria (Holloway et al., 2007) and likely destroys a disproportionate amount of the weakest mitochondria where any inherent dysfunction exists. In addition, the mitochondrial morphology is severely compromised using these techniques. By permeabilizing the outer cell membrane with a gentle detergent and measuring oxygen consumption in a respiration chamber in the presence of various substrates, mitochondrial metabolism can be measured while avoiding all of these concerns.

Previously we have shown that impaired lipid oxidation observed in skeletal muscle of obese individuals, is retained in primary muscle cells cultures (Hulver et al., 2005). By isolating satellite cells from obese humans, we are able to examine skeletal muscle-specific responses to experimental manipulations and eliminate interference from other tissues or circulating factors that may be contributing to the etiology of obesity and impairments in skeletal muscle metabolism.

By bringing these techniques together, we have developed a new model for assessing skeletal muscle mitochondrial metabolism. We will measure mitochondrial respiration rates of permeabilized cultured myotubes from both lean and obese individuals in the presence of various substrates, including palmitoyl carnitine and pyruvate. In addition we will determine mitochondrial content using various methods (mitochondrial DNA copy number, COX-IV protein content). We hypothesize that any phenotypic differences between cells from lean and obese individuals would be exacerbated by the lipid pre-incubation, and that differences in mitochondrial content will not be solely responsible for these differences.

Summary. Controversy exists over whether the obese/type 2 diabetic phenotype is characterized by mitochondrial dysfunction, decreased mitochondrial content, or a combination of both. Using a novel model of phenotypic skeletal muscle metabolism we will investigate these uncertainties.

CONCLUSION

Understanding skeletal muscle substrate selection and the observed oxidation limitation in obese individuals is pertinent to the treatment of the obesity epidemic in the United States. Data suggesting that obese individuals have an inability to alter substrate utilization in the face of a metabolic challenge, such as a carbohydrate load or insulin stimulation, leaves many unanswered questions as to how obese individuals would metabolize a lipid load. In the first study performed in this dissertation, we tested the hypothesis that, while the skeletal muscle of lean individuals will respond to a short-term, high fat diet with increased mRNA content of genes associated with lipid oxidation, the skeletal muscle of obese individuals will not upregulate these genes to a similar extent, indicating impaired mitochondrial function and metabolic inflexibility.

In order to more closely examine potential metabolic anomalies in the skeletal muscle of obese individuals, the purpose of the second study of this dissertation was to assess mitochondrial content, activity, and cellular respiration of myotubes cultured from lean and obese individuals, both with and without a 24h lipid challenge. Based on preliminary data, we hypothesized that permeabilized cells from obese individuals would exhibit lower respiration in all conditions. Also, permeabilized cells from lean men will have greater respiration after palmitate incubation, though cells from obese individuals will not respond to this lipid challenge. We hypothesized that lean individuals will respond to 24h lipid exposure by oxidizing less pyruvate, whereas cells

from obese will not exhibit this shift in metabolic fuel selection. Any defects in cellular respiration observed in the cells from the obese individuals can either be explained by a lower total mitochondrial content or a lower oxidative enzyme activity that is preserved in satellite cells. Finally, if metabolic inflexibility is observed is these cells cultures, it could be hypothesized that the nature of the defect is either of a genetic or epigenetic origin.

CENTRAL HYPOTHESIS

The ability to regulate lipid oxidation in response to lipid exposure (metabolic flexibility) is impaired in skeletal muscle of obese individuals (metabolic inflexibility) and is an important aspect of the mitochondrial dysfunction observed with obesity. This hypothesis will be tested with the following specific aims.

SPECIFIC AIM #1

Whole body lipid oxidation increases in response to high fat feeding in lean humans. However, the ability to switch substrate utilization in response to this metabolic challenge may be impaired with obesity. We determined if the mRNA content of genes involved with lipid oxidation was compromised in response to a high fat diet extremely obese compared to lean individuals.

a. Are genes involved in lipid metabolism activated similarly in skeletal muscle from lean and extremely obese individuals following a high fat meal? Muscle biopsies

were taken from lean and extremely obese individuals in the fasted state and following a high fat meal. mRNA was extracted from muscle tissue and analyzed for genes involved in lipid metabolism (PDK4, CPT1, UCP3, PGC-1α, PPARα, PPARδ, and PPARγ).

b. <u>Are genes involved in lipid metabolism activated similarly in skeletal muscle from lean and extremely obese individuals following 5 days of a high fat diet?</u> Muscle biopsies were taken from lean and extremely obese individuals in the fasted state and before and after 5 days of a high fat diet. mRNA was be extracted from muscle tissue and analyzed for genes involved in lipid metabolism (PDK4, CPT1, UCP3, PGC-1α, PPARα, PPARδ, and PPARγ). The proposed experiment was designed to determine if a more prolonged exposure to lipid *in vivo* equally upregulates genes associated with lipid metabolism in lean and extremely obese individuals.

SPECIFIC AIM #2

Lipid oxidation is impaired in skeletal muscle and cultured myotubes from lean and extremely obese individuals. By permeabilizing cultured myotubes and using high resolution respirometry, we are able to assess respiration in response to different substrates, activators, and inhibitors in cells from lean and extremely obese donors *in situ*. The purpose of these studies was to determine if mitochondrial respiration is

impaired in cells from extremely obese compared with lean individuals and whether this is affected by 24h lipid pre-incubation. By utilizing various experimental conditions including respiration in the presence of palmitoyl carnitine, pyruvate and FCCP we attempted to determine if potential differences in respiration are associated with decreased mitochondrial content or simply impaired mitochondrial function. It is our hypothesis that cells from extremely obese individuals exhibit lower respiration rate than cells from leans, and 24h lipid preincubation will increase respiration in the cells from lean donors. This same effect will not be observed in the cells from the obese, indicating metabolic inflexibility.

a. Is mitochondrial respiration impaired in cultured myotubes from lean and extremely obese individuals and is it affected by 24h of lipid preincubation? Muscle biopsies were taken from lean and extremely obese individuals in the fasted state and cultured into myotubes. Upon differentiation, cells were incubated for 24h in either lipid or control media. Cells were then made permeable to substrates and respiration measured in the presence of different substrates, including palmitate and pyruvate.
b. Is the impaired respiration observed in cultured myotubes from extremely obese individuals due to detriments in mitochondrial content? Muscle biopsies were taken from lean and extremely obese individuals in the fasted state and satellite cells cultured into myotubes. Upon differentiation, cells were incubated with

lipid for 24h and used to determine mitochondrial DNA copy number, citrate synthase and β-HAD activity, and COX-IV protein content.

CHAPTER 2: SKELETAL MUSCLE METABOLIC INFLEXIBILITY IS EVIDENT WITH HIGH FAT FEEDING IN OBESE HUMANS

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Keywords: Skeletal Muscle, PDK4, Metabolic Inflexibility

Abstract: In lean individuals, increased dietary lipid results in increased whole body lipid oxidation; however, this capacity to respond to substrate availability (i.e. metabolic flexibility) appears to be compromised with obesity. The purpose of the present study was to determine if the responses of genes linked with lipid oxidation were altered with exposure to dietary lipid in the skeletal muscle of obese humans. Lean (BMI = 22.1 ± 0.6 kg/m^2) and obese (BMI = 39.6 ± 1.7 kg/m^2) individuals were studied before and after a 5 d high fat diet (65% of total energy from fat). Skeletal muscle biopsies (vastus lateralis) were obtained in the fasted and fed states before and after the intervention and mRNA content for genes involved in lipid oxidation determined. Fasted PDK4 mRNA content

increased by 2-fold in the leans (P<0.05), but tended to decrease in the obese (-46%, P = 0.07) following the HFD. Fasting UCP3 content fell by half in the obese from Pre- to Post-HFD ($P < 0.05$), but did not change in the leans. Multivariate analysis revealed a similar elevation in the leans and/or a decrease in the obese subjects for many genes that control lipid oxidation following the HFD (PDK4, UCP3, CPT1, PPARα, PPARγ, PPARδ, and PGC-1α). As a functional measure, medium chain lipid species decreased from Pre- to Post-HFD in the lean participants ($P < 0.05$), but did not change in the obese individuals. These data suggest an inability to respond to a lipid stimulus that can be extended to gene regulation in skeletal muscle tissue of obese humans which is indicative of a global aberration in lipid metabolism.

INTRODUCTION

Obesity rates in the United States have recently doubled in adults and tripled in children (Ogden et al., 2006). An important facet of this obesity epidemic is understanding skeletal muscle lipid metabolism, particularly in light of the elevated lipid content (Goodpaster et al., 2000) and impaired ability to oxidize lipids in the skeletal muscle of obese individuals (Hulver et al., 2003; Kim et al., 2000). In healthy, lean individuals, skeletal muscle lipid oxidation increases in response to fasting and exercise, thereby preserving glucose and glycogen stores, whereas rates of lipid oxidation decline in the postprandial and insulin-stimulated states (Andres et al., 1956; Kelley et al., 1990). This ability to rapidly transition substrate utilization depending upon substrate availability and the hormonal milieu has been termed 'metabolic flexibility' and an inability to appropriately respond to these stimuli has been reported with obesity and type 2 diabetes (for review, see Kelley and Mandarino (2000)).

In reference to metabolic flexibility in lean individuals, a high fat diet can increase whole body lipid oxidation at rest (Buemann et al., 1998; Smith et al., 2000) and during exercise (Cameron-Smith et al., 2003); however, overweight individuals do not display a similar adaptation (Thomas et al., 1992). In support of an inability to respond appropriately to lipid substrate, previously obese individuals and those with a family history of obesity exhibit a blunted increase in lipid oxidation following high fat feeding (Astrup et al., 1994; Brown et al., 2000). These studies indicate that the lipid-

induced metabolic flexibility observed at the whole body level with lipid exposure in lean individuals may be impaired with obesity.

High fat feeding-induced increases in lipid oxidation in lean individuals are accompanied by skeletal muscle-specific increases in PDK4 mRNA content and/or enzyme activity within several days (Arkinstall et al., 2004; Pehleman et al., 2005; Peters et al., 2001), suggesting that lipid exposure results in a rapid induction of genes regulating lipid oxidation in this tissue. To our knowledge, skeletal muscle-specific responses to dietary lipid exposure and have not been investigated in obese humans. The hypothesis to be tested in the current investigation is that a lipid stressor (high fat diet) does not induce transcription of genes linked with lipid oxidation, such as PDK4 and the PPAR family, in the skeletal muscle of obese individuals to the same extent as in muscle of lean participants.

METHODS

EXPERIMENTAL DESIGN AND SUBJECTS

The effect of a single high fat meal and a 5d high fat diet (HFD) on skeletal muscle gene expression was compared between lean (n=12, 9 men and 3 women; BMI ≤ 24.9 kg/m^2) and obese (n=10, 8 men and 2 women; BMI ≥ 35 kg/m^2) Caucasian men and women (ages 18-27 y). Participants were free from disease, nonsmokers, and not taking medications known to alter carbohydrate or lipid metabolism; characteristics are

presented in Table 1. Females participated during the follicular phase of the menstrual cycle (days 1-10). All participants had maintained a constant body mass (± 2 kg) in the 6 months prior to the experiment. The protocol was approved by the East Carolina University Policy and Review Committee on Human Research, and informed consent was obtained.

Participants underwent 5 consecutive days of a eucaloric, high-fat diet (HFD) with assessment of parameters in the fasted state and following a single high fat meal on day 1 and day 6. On day 1 , participants reported to the laboratory between 0630h and 0800h following a 12 hour overnight fast. Body mass was recorded, a venous blood sample was obtained, and a skeletal muscle biopsy was performed. Plasma was separated for subsequent analyses of glucose (YSI 2300 STAT Plus Glucose and Lactate Analyzer, YSI Inc.; Yellow Springs, OH), insulin (Access Immunoassay System, Beckman Coulter; Fullerton, CA), and non-esterified fatty acids (NEFA; Wako Chemicals; Richmond, VA). β-hydroxybutyrate (Pointe Scientific; Canton, MI) was determined from the fasting plasma samples as an index of participant adherence to the HFD. A homeostasis model assessment (HOMA) value (fasting glucose ($mg \cdot dL^{-1}$) x 0.05551) x fasting insulin ($\mu U \cdot mL^{-1}$)/22.1) was calculated (Bonora et al., 1998). Participants were then fed a high-fat meal and asked to return to the laboratory 4 hours later, at which time another venous blood sample and muscle biopsy were obtained. Participants did not eat or drink anything except water during the 4 hours between biopsies.

Participants then consumed the provided HFD for 5 consecutive days. Adherence was assessed by food diary and plasma ketone body analysis as well as communication with the subjects during the 5 day period. On experimental day 6, participants reported to the laboratory and repeated the assessments of day 1.

Plasma samples were also used to determine circulating acylcarnitine profiles as previously described (Koves et al., 2005; Koves et al., 2008).

DIETARY INTERVENTION

The HFD contained 65% of energy from fat, 15% of energy from protein, and 25% of energy from carbohydrate. Daily caloric requirements were based on body size using the Harris-Benedict equation (Harris and Benedict, 1918). The high-fat meal on days 1 and 6 was designed to contain 65% of energy from fat and comprise 35% of each subject's daily energy intake. All meals consisted of pre-measured and prepared meals, which were provided to the participants. Subjects were asked to adhere to the planned menu, to maintain their normal physical activity levels, and to refrain from alcohol consumption for the duration of the study. All diet records, meals, and the high fat diets were analyzed using Nutritionist Pro™ Nutrition Analysis Software (Axxya Systems LLC; Stafford, TX) to assure proper energy intake and macronutrient composition. Subjects performed a 3-day dietary recall prior to performing the study to ensure that they were consuming a normal, mixed diet.

MUSCLE ANALYSES

Skeletal muscle was obtained from the vastus lateralis and total RNA isolated from ~30 mg of frozen muscle using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) with on-column DNase digestion using the RNase-Free DNase Set (Qiagen Inc.; Valencia, CA) to remove residual DNA. RNA was reverse transcribed into cDNA using the Superscript III Reverse Transcriptase protocol (Invitrogen Corp.; Carlsbad, CA) and quantified in triplicate with Quant-iT PicoGreen reagents (Invitrogen Corp.; Carlsbad, CA). Real-time quantitative PCR (RTQ-PCR) was performed using the ABI PRISM 7400HT Sequence Detection System instrument and software with Taqman® Universal PCR Master Mix in accordance with manufacturer's instructions (Applied Biosystems Inc. (ABI); Foster City, CA). Relative gene expression levels were determined using the number of cycles necessary to reach threshold (Ct). The Ct values from RTQ-PCR were compared with a standard curve consisting of a serially diluted pool from each of the samples. All samples from each subject were run on the same plate and values normalized to total cDNA. Endogenous control gene expression (18S) was measured and compared with each gene of interest to assure assay efficiency. Primer and probe sequences are presented in Table 2. These particular genes were measured because of their explicit roles in lipid metabolism and the PPAR/PGC-1α regulatory system.

Approximately 20 mg of muscle tissue was processed for acylcarnitine profiling as an index of substrate utilization. Samples were centrifuged and processed as

previously described (Koves et al., 2005). Acylcarnitines were measured by direct-injection electrospray tandem mass spectrometry, using a Micromass Quattro Micro™ system equipped with a model 2777 autosampler, a model 1525 high pressure liquid chromatography solvent delivery system, and a data system running 4.0 MassLynx software (Waters; Milford, MA).

Statistical Analyses

Repeated measures analyses of variance were used to compare the data. Significant interactions were examined using *post-hoc* contrast-contrast comparisons. A multivariate analysis (body size x HFD) was used to compare changes among genes associated with lipid oxidation between lean and obese. Although fold change data are presented in some figures for ease of visualization, statistical analyses were performed on raw data. Statistical significance was denoted at the $P < 0.05$ level and all data are presented as the mean ± SEM.

Results

Diet and Plasma Analysis

The HFD provided a similar macronutrient composition for each group (Lean: 62.2% fat, 22.6% carbohydrate, 14.9% protein; Obese: 61.9% fat, 22.2% carbohydrate, 15.1% protein); the HFD provided a significant increase in relative dietary fat intake over

normal consumption, as reported by a 3d diet record (Lean: 36% fat, Obese: 36% fat) with no change in energy intake. Fasting β-hydroxybutyrate levels increased in both groups as a result of the dietary intervention (1.6 fold increase, $P < 0.05$) indicating adherence to the HFD. Blood glucose levels were not different between the groups (Table 3). NEFA values were not different between lean and obese subjects and were not affected by the diet intervention. However, the obese group displayed elevated fasting insulin concentrations and HOMA compared to the leans (Table 3).

MUSCLE ANALYSES

In response to the high fat meal on day 1, there was a significant interaction between lean and obese individuals for PPARα, where gene expression was elevated in the Post-Meal state in the leans (3-fold increase, $P < 0.05$), but not in the obese (Figure 1).

Multivariate analysis revealed a significant body size x HFD interaction for many genes involved in lipid oxidation (PDK4, UCP3, CPT1, PPARα, PPARγ, PPARδ, and PGC-1α; $P < 0.05$, Figure 2), indicating disparate responses in gene regulation following the 5 d HFD in lean and obese humans. Specifically, body size x HFD interactions were noted for PDK4, UCP3, PPARα, and PPARγ ($P < 0.05$), though only PDK4 and UCP3 had significant *post-hoc* comparisons. Fasting PDK4 mRNA content increased (2-fold increase, $P < 0.05$) in the leans with the HFD but tended to decrease in the obese (-46%, $P = 0.07$) subjects,

resulting in lower PDK4 mRNA content in obese compared with leans after the HFD ($P < 0.05$, Figure 3). Fasting UCP3 mRNA content fell in the obese from Pre- to Post-HFD (-46%, $P < 0.05$) but did not change in the leans (+15%, n.s., Figure 3).

The Post-Meal tissue samples on days 1 and 6 of the HFD were used to assess differences in 37 skeletal muscle acylcarnitine species as an index of metabolic flux through lipid oxidation (Koves et al., 2008). There was a significant body size x HFD interaction for all medium chain molecules combined (-42%, $P < 0.05$; and +17%, n.s.; for lean and obese; respectively) as well as for several medium chain species (C4/Ci4, C6, C10:1, C10, C8:1DC, and C12:1; n = 6 lean, 6 obese) where the lipid intermediates C10:1, C10, C8:1DC, and C12:1 decreased in the lean participants and C8:1DC increased in the obese from Pre- to Post-HFD ($P < 0.05$, Figure 4). Long chain species were not different between lean and obese and decreased in both groups after the HFD (-48% and -45% for lean and obese, respectively; $P < 0.05$). Neither medium chain nor long chain species were significantly different between lean and obese at baseline.

Fasting insulin values were inversely correlated ($P < 0.05$) with changes in fasted mRNA content of PDK4 ($r^2 = 0.29$), CPT1 ($r^2 = 0.28$), UCP3 ($r^2 = 0.31$), and PGC-1α ($r^2 = 0.47$). BMI was only correlated with changes in PDK4 mRNA content ($r^2 = 0.27$, $P = 0.05$).

DISCUSSION

Whole body lipid oxidation increases in response to high fat feeding in lean humans (Astrup et al., 1994; Buemann et al., 1998; Cameron-Smith et al., 2003; Peters et al., 2001; Schrauwen et al., 2000; Smith et al., 2000). However, other findings suggest that obese or previously obese individuals do not adapt to lipid exposure in similar manner (Astrup et al., 1994; Thomas et al., 1992). Based on these reports the goal of the present study was to test the hypothesis that genes associated with lipid oxidation would increase in the skeletal muscle of lean individuals with a 5 day high fat diet (metabolic flexibility), but remain unaltered in obese individuals. The data obtained support this hypothesis, as a multivariate analysis indicated skeletal muscle of lean and obese responded differently to dietary lipid exposure that was evident in genes involved with lipid oxidation (Figure 2). The findings of the present study thus provide the novel information that the inability to appropriately respond to a nutritional stimulus ('metabolic flexibility') (Kelley et al., 1999; Kelley and Mandarino, 2000; Koves et al., 2008) can be extended to gene regulation in the skeletal muscle of obese humans. Such findings are indicative of a relatively global dis-regulation of lipid oxidation with obesity.

The current study investigated the changes in mRNA content of the PPARs and PPAR-responsive genes as indicators of a global regulatory system involved in lipid metabolism. In particular, PPARα and PGC-1α are provocative candidates for a 'master regulator' given their relative abundance in skeletal muscle and that these proteins are

targeted by lipids and lipid derivatives to induce transcription of genes involved in lipid oxidation such as PDK4, CPT1, and UCP3 (Koves et al., 2005; Muoio et al., 2002).

PPARα increased with acute lipid intake after the high fat meal on day 1 in the lean but not obese participants (Figure 1), an interaction that was carried over to the fasted state on day 6 (Figure 2). These data suggest that PPARα may have some regulatory role in lipid-inducible substrate selection in lean individuals but that this response is dampened or even eliminated in the skeletal muscle of obese subjects. However, after our 5 d HFD, distinct increases in fasting PPARα mRNA content in lean men were not observed, similar to previous reports (Chokkalingam et al., 2007; Helge et al., 2007). Thus, the present findings indicate that PPARα may respond acutely to lipid oversupply in terms of upregulating gene expression but that a longer-term adapation is not evident. In terms of PGC-1α, Sparks et al. (2005) found a 20% decrease in fasting PGC-1α mRNA content in lean men after a three day, 50% fat diet which is not consistent with our findings (Figure 2). The authors suggest that HFD-induced insulin resistance may have reduced PGC-1α gene expression (Sparks et al., 2005); we did not observe changes in fasting insulin, glucose or HOMA throughout our protocol, which may explain why we did not observe a similar decrease in PGC-1α. Although mRNA content was determined, both PGC-1α and PPARα activity are also governed by post-transcriptional regulation (ligand, coactivator, and DNA binding) which could also be a factor accounting for the upregulation of genes linked with lipid oxidation in the lean

subjects (Figure 2) (Desvergne and Wahli, 1999; Puigserver et al., 1999). Nevertheless, both PGC-1α and PPARα displayed a similar pattern of response to other genes involved in lipid oxidation in that there was increased expression in lean and/or decreased expression in the skeletal muscle of obese individuals.

PDK4 was examined as potentially the most potent nutritional responder and target gene of the PPAR/PGC-1α regulatory system; in addition, PDK4 plays a critical role in the partitioning of substrate towards either lipid or carbohydrate oxidation (Sugden and Holness, 2006). The lean participants exhibited a 2-fold increase in PDK4 mRNA content with the 5 days of the HFD in accordance with previous reports (Chokkalingam et al., 2007; Sparks et al., 2006) (Figure 3). Consistent with the concept of metabolic inflexibility in obese individuals, we hypothesized that PDK4 mRNA content would remain unaltered with the intervention; however, we were surprised to find a trend toward *decreased* PDK4 mRNA content with the HFD ($P = 0.07$, Figure 2). HFD-induced increases in PDK4 mRNA content in lean subjects are generally accompanied by corresponding increases in transcribed PDK4 protein (Cameron-Smith et al., 2003; Peters et al., 2001), decreases in PDH activation (Chokkalingam et al., 2007; Pehleman et al., 2005) and increased whole body lipid oxidation (Cameron-Smith et al., 2003; Peters et al., 2001). Hence, changes in skeletal muscle PDK4 mRNA content with high fat feeding in lean subjects appear to have significant physiological effects that are consistent with changes in lipid oxidation observed in the whole body.

Among lean individuals, Smith and colleagues (2000) noted that those with the most robust HFD-induced increases in lipid oxidation also had the lowest fasting insulin levels. Similarly, we noted that those with the lowest fasting insulin values exhibited the largest changes in PDK4 mRNA content ($r^2 = 0.29$; $P < 0.05$). Such findings suggest that perhaps the consistently elevated insulin values in our obese participants suppressed any potential lipid-induced increase in PDK4 gene expression. However, we did not observe changes in fasting insulin concentration as a result of our intervention and PDK4 mRNA values were not depressed in the obese subjects prior to the HFD despite elevated fasting insulin levels (Figure 2 and Table 3). In addition, changes in PDK4 were also inversely correlated with BMI ($r^2 = 0.27$; $P < 0.05$), so a clear distinction between these factors cannot be made.

Physiological adaptation to a lipid challenge, as evidenced by fat balance (respiratory quotient/dietary lipid quotient), can occur within 6-7 days in lean humans (Peters et al., 2001; Schrauwen et al., 2000). By day five of our HFD, changes in PDK4 mRNA content in our lean participants were consistent with metabolic adaptation. However, obese individuals may take longer to adapt, or may not at all; which provides a mechanism for increased susceptibility to skeletal muscle lipid accumulation and dietary-induced obesity.

To gain further insight into this dysregulation of fat oxidation in obesity, we evaluated a panel of 37 acylcarnitine species in the Post-Meal tissue samples. An

accumulate of even chain acylcarnitine species ranging from C6-C22 indicate incomplete fatty acid oxidation, while a reduction reflects a higher rate of complete lipid oxidation (Koves et al., 2005). In accordance with the mRNA data, the acylcarnitine findings (Figure 4) suggest improper lipid handling in the obese individuals which was largely unaltered with the high fat diet. In contrast, the lean subjects appeared to increase the capacity for the complete oxidation of lipids with the HFD. Medium chain skeletal muscle acylcarnitines decreased after the HFD in the leans, suggesting more efficient lipid handling, which is consistent with HFD-induced increases in whole body lipid oxidation in lean (Buemann et al., 1998; Cameron-Smith et al., 2003; Peters et al., 2001; Smith et al., 2000). The observed increases in medium chain species in the obese individuals (Figure 4) suggests reduced TCA-cycle capacity, as has been previously described with obesity and high fat feeding (Koves et al., 2008). Alternatively, medium, but not long, chain species accumulation could indicate reduced lipid oxidation though MCAD, which appears to be regulated via PGC-1α in conjunction with high fat feeding (Koves et al., 2005). Indeed, elevated C8 acylcarnitine species, as observed in the obese individuals after the HFD (Figure 4), are indicative of MCAD deficiency which may be accompanied by elevated C6, C10, and C10:1 species (Rinaldo et al., 2008). Either interpretation suggests a mechanism of lipid accumulation linked to mitochondrial overload and incomplete fatty acid oxidation which manifests as metabolic inflexibility with obesity (Koves et al., 2008).

Excess skeletal muscle lipids, particularly those in or near the mitochondria, are prone to oxidation by reactive oxygen species (ROS), leading to accumulation of damaging lipid peroxides, thus reducing mitochondrial oxidative capacity (Schrauwen, 2007). Likewise, we have recently reported that skeletal muscle fibers from obese men had higher basal H_2O_2 emission than leans, indicative of a more oxidized cellular environment (Anderson et al., 2009). In the current study, the HFD decreased UCP3 mRNA content by almost half in the obese participants (Figure 2). This response may be critical to mitochondrial function, as UCP3 has been reported to aid in ameliorating lipid-induced mitochondrial damage by buffering lipid peroxide accumulation (Schrauwen, 2007) and decreasing H_2O_2 emission (Anderson et al., 2007). The present findings thus suggest that obese individuals may not be able to induce this protective effect from UCP3 in the face of increased lipid intake, which may lead to mitochondrial damage and the reduced oxidative capacity observed in skeletal muscle of obese individuals (Kelley et al., 2002; Ritov et al., 2005; Simoneau et al., 1999).

In conclusion, five days of high fat feeding coordinately upregulates genes involved in lipid oxidation in the skeletal muscle of lean individuals. In contrast, obese individuals fail to respond and, in some instances, exhibit decreased mRNA content of these same genes. These data suggest an inability to respond to a lipid stimulus that can be extended to gene regulation in skeletal muscle tissue of obese humans which is indicative of a global aberration in lipid metabolism.

TABLE 1. PARTICIPANT CHARACTERISTICS.

All data are presented as mean ± SEM. *Indicates significant difference between the groups ($P < 0.05$).

	Lean (n=12)	Obese (n=10)
Age (y)	21.8 ± 0.7	22.0 ± 0.9
Stature (cm)	174.1 ± 2.5	181.9 ± 2.3
Mass (kg)	67.0 ± 2.7	130.8 ± 5.4*
BMI (kg/m^2)	22.1 ± 0.6	39.6 ± 1.7*
Glucose (mmol/L)	4.92 ± 0.14	4.57 ± 0.19
Insulin (µU/L)	5.9 ± 1.1	16.3 ± 1.6*
HOMA	1.6 ± 0.3	3.6 ± 0.4*
Cholesterol (mg/dL)	163.1 ± 8.3	163.8 ± 13.8
Triglycerides (mg/dL)	113.1 ± 12.7	131.9 ± 26.5

TABLE 2. PRIMER AND PROBE SEQUENCES.

Primer and Probe sequences used to determine mRNA content analyses using RT-PCR, including PDK4, UCP3, CPT1, PGC-1α, PPARα, PPARδ, and PPARγ. Sequences are listed in 5' to 3' orientation.

	Forward Primer	Reverse Primer	Probe
PDK4	TCCACTGCACCAACGCCT	TGGCAAGCCGTAACCAAAA	ATAATTCCCGGAATGCTCCTTTGGCTG
UCP3	TGACTCCGTCAAGCAGGTGTAC	CAAAATCCGGGTAGTGAGGCT	CCCCCAAAGGCGCGGACAAC
CPT1	CTGCAGTGGGACATTCCAAA	CAACGCCTTGGCCACCT	ACTCTCGATGACCGCCTGGCACTG
PGC-1α	CAAGCCAAACCAACAACTTTATCTCT	CACACTTAAGGTGCGTTCAATAGTC	AGTCACCAAATGACCCCAAGGGTTCC
PPARα	GCAACCACCCGGACGATA	GCCGGAGGTCTGCCATTT	CTTTCTCTTCCCAAAACTTCTTCAA
PPARδ	AGCATCCTCACCGGCAAA	GTCTCGATGTCGTGGATCACA	CCAGCCACACGGCGCCCT
PPARγ	GGCTTCATGACAAGGGAGTTTC	AACTCAAACTTGGGCTCCATAAAG	AAAGAGCCTGCGAAAGCCTTTTGGTG

TABLE 3. PLASMA ANALYSIS.

* indicates a significant difference between lean and obese. [a] indicates a significant difference between Fasted and Post-Meal values in both lean and obese combined. [b] indicates a significant difference between Fasted and Post-Meal values in the obese group. All data are presented as mean ± SEM.

	Lean (n=12)	Obese (n=10)
Glucose (mmol/L)		
Pre HFD Fasted	4.74 ± 0.12	4.78 ± 0.17
Pre HFD Post-Meal	4.54 ± 0.13[a]	4.33 ± 0.14[a]
Post HFD Fasted	4.46 ± 0.18	4.80 ± 0.09
Post HFD Post-Meal	4.50 ± 0.10	4.10 ± 0.69
Insulin (µU/L)		
Pre HFD Fasted	4.63 ± 0.52	14.7 ± 2.9*
Pre HFD Post-Meal	4.97 ± 0.54	14.1± 3.8*
Post HFD Fasted	4.81 ± 0.66	11.5 ± 1.6*
Post Diet HFD Post-Meal	5.49 ± 0.85	14.5 ± 3.0*[b]
NEFA (mEq/L)		
Pre HFD Fasted	0.66 ± 0.13	0.49 ± 0.07
Pre HFD Post-Meal	0.50 ± 0.03	0.71 ± 0.15
Post HFD Fasted	0.71 ± 0.08	0.56 ± 0.10
Post Diet HFD Post-Meal	0.56 ± 0.05	0.60 ± 0.16

FIGURE 1. MEAL RESPONSE OF PPARα MRNA CONTENT.

Data are PPARα mRNA contents of lean (open bars) and obese (filled bars) individuals in the Fasted and Post-Meal states in the Pre-HFD condition. Data are expressed as arbitrary units. All values are expressed as mean ± SEM. * Indicates significant difference from fasted value. Significance is indicated at $P < 0.05$.

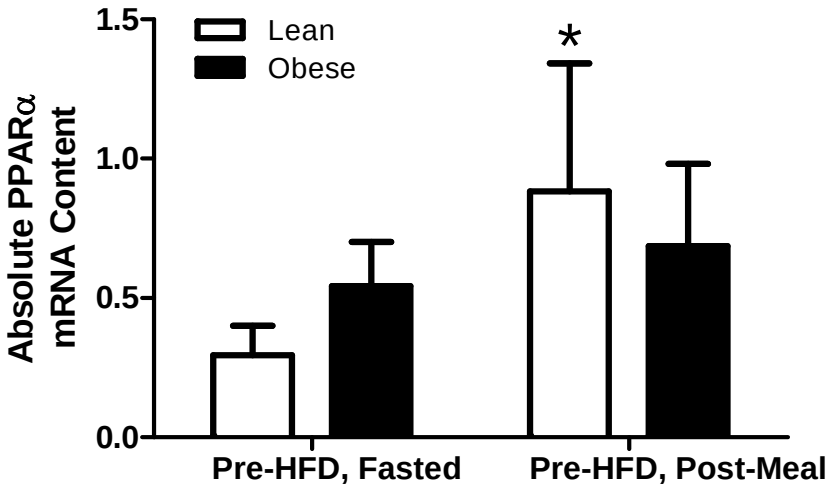

1.5
1.0
0.5
0.0
Absolute PPARα mRNA Content
Lean
Obese
*
Pre-HFD, Fasted
Pre-HFD, Post-Meal

FIGURE 2. EFFECT OF HFD ON MRNA CONTENT IN THE FASTED STATE.

Change in mRNA content with the 5-day HFD diet in the skeletal muscle of lean (open bars) and obese (filled bars) individuals. Values are expressed as the fold change from the Pre- to Post-HFD conditions where the Pre-HFD; no change with the HFD would then be indicated by a value of 1 which is presented by the dashed line. Values are mean ± SEM. The overall interaction for all genes in the Pre- and Post-HFD conditions by body size was significant as determined by multivariate analysis, indicating a differential response between the lean and obese subjects.

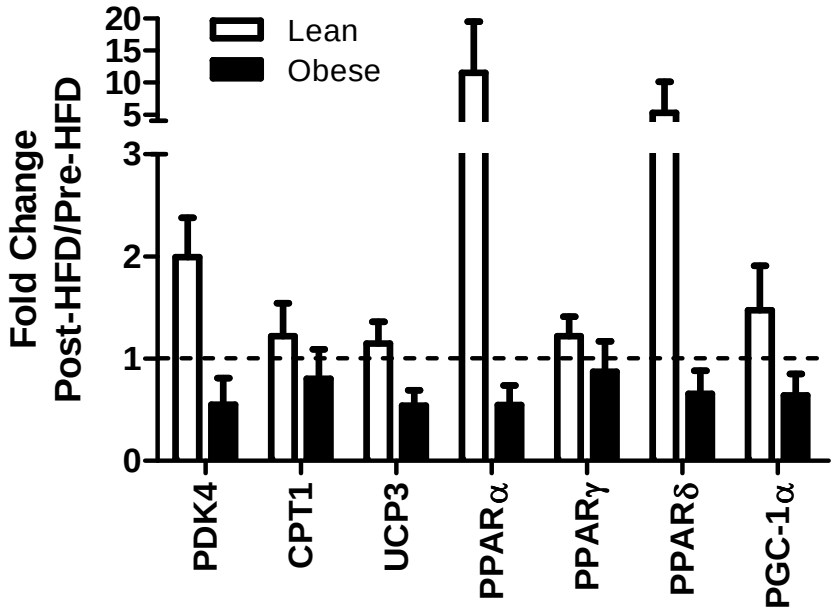

Fold Change
Post-HFD/Pre-HFD
20
15
10
5
3
2
1
0
Lean
Obese
PDK4
CPT1
UCP3
PPARα
PPARγ
PPARδ
PGC-1α

FIGURE 3. RESPONSE OF PDK4 AND UCP3 MRNA CONTENT TO 5-DAY HFD.

PDK4 (Panel A) and UCP3 (Panel B) mRNA contents of lean (open bars) and obese (filled bars) individuals in the Fasted state in the Pre- and Post-HFD condition. Data are expressed as arbitrary units. All values are expressed as mean ± SEM. * Indicates significant difference from Pre-HFD condition. # Indicates significant difference from lean in the Post-HFD condition. Significance is indicated at $P < 0.05$.

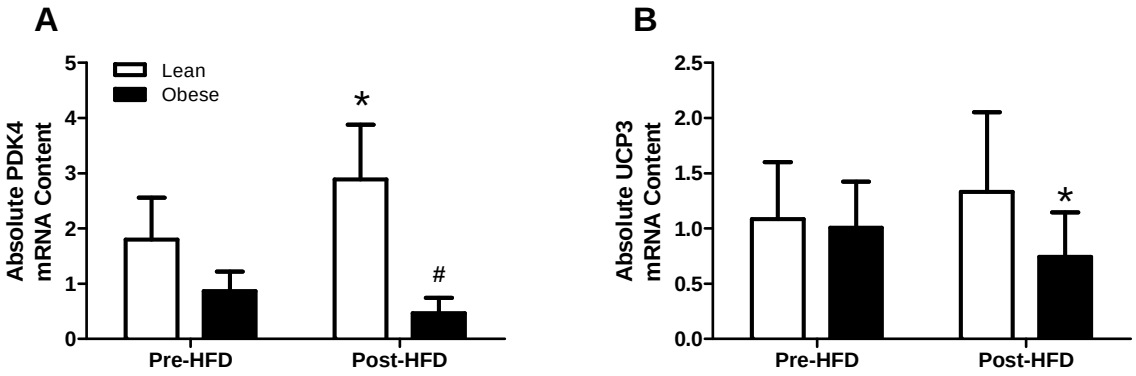
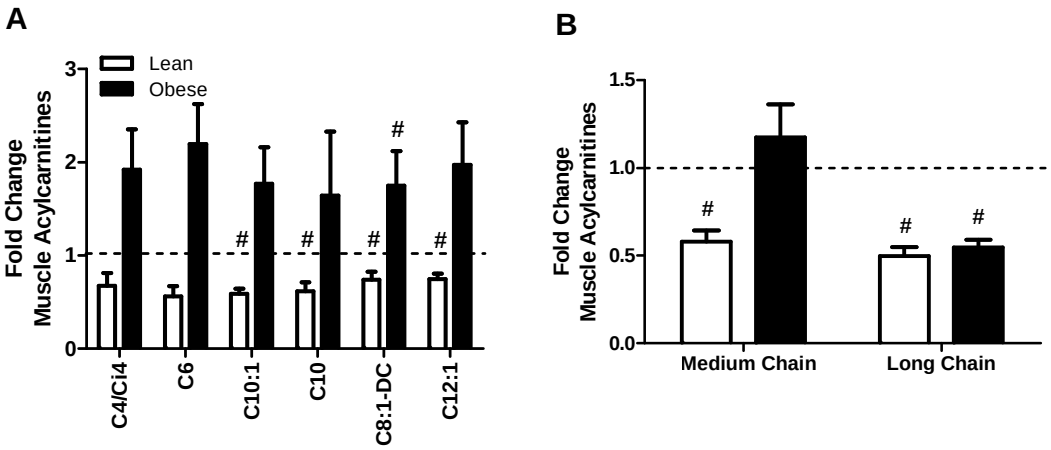

A
Lean
Obese
Absolute PDK4 mRNA Content
5
4
3
2
1
0
*
#
Pre-HFD
Post-HFD
B
Absolute UCP3 mRNA Content
2.5
2.0
1.5
1.0
0.5
0.0
*
Pre-HFD
Post-HFD

FIGURE 4. EFFECT OF HFD ON MUSCLE ACYLCARNITINES IN THE POST-MEAL STATE.

Data are medium chain muscle acylcarnitines showing a significant body size x HFD interaction (Panel A) in lean (open bars) and obese (filled bars) individuals in the Post-Meal state in the Pre- and Post-HFD condition. Fold change in medium and long chain acylcarnitine species in lean and obese are presented in Panel B, no change with the HFD would then be indicated by a value of 1 which is presented by the dashed line. A value > 1.0 indicates net accumulation of a acylcarnitine species while a value < 1 indicates more fewer incompletely oxidized lipids. All values are expressed as mean ± SEM. # Indicates significant difference from Pre-HFD value. Significance is indicated at $P < 0.05$.


A
Lean
Obese
Fold Change
Muscle Acylcarnitines
3
2
1
0
#
C4/Ci4
C6
C10:1
C10
C8:1-DC
C12:1
B
Fold Change
Muscle Acylcarnitines
1.5
1.0
0.5
0.0
Medium Chain
Long Chain

CHAPTER 3: MITOCHONDRIAL FUNCTION IS IMPAIRED IN CULTURED MYOTUBES FROM OBESE HUMANS

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Keywords: Skeletal Muscle, Metabolic Inflexibility

Abstract:

The skeletal muscle of obese humans is characterized by reduced oxidative capacity and an inability to appropriately respond to a metabolic stimulus. The purpose of the current study was to determine if this metabolic inflexibility is evident in intact skeletal muscle cells (myotubes) derived from lean and obese donors and to identify possible mechanisms involved. Respiration was determined in permeabilized cultured myotubes from lean and obese individuals before and after a 24 h lipid incubation. Indicative of metabolic inflexibility with obesity, state 3 and uncoupled respiration rates with lipid (palmitoyl carnitine) and carbohydrate (pyruvate) substrate increased by up to 2-fold after the 24 h lipid incubation in myotubes from lean, but not obese, donors. The 24 h lipid incubation increased mitochondrial DNA (mtDNA) content in myotubes from

lean subjects by 16% ($P < 0.05$), conversely, mtDNA content tended to decrease by 13% in myotubes from obese individuals ($P < 0.06$). Elevated respiration in the cells from lean individuals after 24 h lipid incubation was still evident when data were normalized to mitochondrial content, suggesting that factors other than mitochondrial proliferation were involved. In summary, these data suggest that the skeletal muscle of obese individuals is metabolically inflexible in terms of increasing substrate oxidation in response to lipid exposure. This decrement is evident in primary cell culture and may involve, at least in part, an inability to induce mitochondrial proliferation.

INTRODUCTION

Skeletal muscle lipid oxidation is reduced in obese and insulin resistant populations as determined in single leg perfusions (Kelley et al., 1999), tissue homogenates (Hulver et al., 2003; Kim et al., 2000), and primary muscle cell culture (Hulver et al., 2005). Likewise, the skeletal muscle of obese humans is characterized by accumulated lipids (Goodpaster et al., 2000) and reduced oxidative capacity (Kelley et al., 1999; Kelley et al., 2002; Menshikova et al., 2005; Ritov et al., 2005). While some suggest that the reduced oxidative capacity in obesity is based largely on differences in mitochondrial content (Boushel et al., 2007; Holloway et al., 2009; Holloway et al., 2007), others argue that the existing mitochondria are unable to effectively oxidize lipids (Kelley et al., 2002; Koves et al., 2008).

Emerging evidence indicates that the ability to adapt to a substrate challenge, such as insulin stimulation or lipid exposure, may be an important aspect of the oxidative impairment in the obese/diabetic phenotype (metabolic flexibility) (Astrup et al., 1994; Kelley et al., 1999). Moreover, studies suggest that lipid exposure may exacerbate these phenotypic differences (Costford et al., 2008; Koves et al., 2008). We have previously reported the retention of reduced lipid oxidation in primary human muscle cell cultures (myotubes) from obese individuals (Hulver et al., 2005). In addition, Wensaas et al. (2009) have noted a less robust increase in palmitate oxidation in cultured myotubes from type 2 diabetics following 4 days of lipid incubation, suggesting

metabolic inflexibility in these cells. However, in these previous studies, lipid oxidation has only been measured in the basal, non-ADP-stimulated state (Hulver et al., 2005; Wensaas et al., 2009) which do not reflect differences in mitochondrial capacity. By permeabilizing the outer cell membrane and measuring mitochondrial respiration in ADP-stimulated and chemically uncoupled states, we are able to determine the maximal oxidative capacity of these cultured myotubes which would provide valuable insight into the etiology of the mitochondrial dysfunction in skeletal muscle of obese individuals.

The purpose of the present study was to determine if metabolic inflexibility is evident in skeletal muscle cells derived from obese donors and, if so, the nature of this defect. Maximal and sub-maximal mitochondrial respiration rates of permeabilized cultured myotubes from both lean and obese individuals were determined after a 24 h lipid pre-incubation in the presence of either lipid (palmitoyl carnitine) or carbohydrate (pyruvate). We hypothesized that any phenotypic differences between cells from lean and obese individuals would be exacerbated by a lipid pre-incubation. Data were normalized to both total cell count and mitochondrial DNA copy number to gain insight into the role of mitochondrial content in the phenotypic differences in skeletal muscle oxidative capacity.

METHODS

DESIGN

Mitochondrial respiration was measured in cultured myotubes from lean and obese donors both with and without a 24 h lipid pre-incubation. Respiration was measured in permeabilized cells in the presence of palmitoyl carnitine and pyruvate as indicators of overall lipid (palmitoyl carnitine) and carbohydrate (pyruvate) flux through Complex I of the mitochondria (Table 5). Succinate was then added to determine oxygen flux through Complex II of the mitochondria.

MATERIALS

All chemical reagents and substrates were purchased from Sigma (St. Louis, MO), unless otherwise stated. Fetal bovine serum (FBS), Heat-inactivated horse serum, gentamicin, 0.05% trypsin EDTA, and Hank's balanced salt solution were from Invitrogen (Carlsbad, CA). Growth media (GM) and differentiation media (DM) consisted of low glucose (5 mmol/L) Dulbecco's modified Eagles medium (DMEM) from Invitrogen. Biocoat tissue culture plates were from Becton Dickinson (Franklin Lakes, NJ). PCR reagents were from Applied Biosystems (Foster City, CA).

HUMAN SUBJECTS

Skeletal muscle biopsies were performed in 7 lean (BMI < 25 kg/m^2) and 8 obese (BMI > 35 kg/m^2) Caucasian males (ages 18-27 y). Participants were free from disease, nonsmoking, and none were taking medications known to alter metabolism. All participants had maintained a constant body mass (± 2 kg) in the 6 months prior to the biopsy. The protocol was approved by the East Carolina University Policy and Review Committee on Human Research, and informed consent was obtained. Subject characteristics are presented in Table 4.

PRIMARY HUMAN SKELETAL MUSCLE CELL CULTURE

Muscle samples weighing 50–100 mg, which were obtained from vastus lateralis by needle biopsy under local anesthesia (0.1% lidocaine) and were immediately transferred to ice-cold low glucose DMEM and cleaned free of adipose and connective tissues. Satellite cells were isolated with 0.25% trypsin collagenase digestion, pre-plated 1-3 h in 3 mL GM on an uncoated T-25 tissue culture flask to remove fibroblasts, and then transferred to a type I collagen-coated T-25 flask for attachment. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in GM supplemented with human skeletal muscle SingleQuot contents, minus insulin (Lonza, Switzerland). After reaching ~70% confluence, myoblasts were transferred to a type I collagen-coated T-75 flask. At ~70% confluence, cells were frozen in aliquots of ~0.5 x 10^6 in 0.5% DMSO for later use. For experiments, cells were thawed and cultured to ~70% confluence in 1 T-

75, then sub-cultured into 2 T-75s. When cells reached 80–90% confluence, differentiation was induced by changing to low-serum DM consisting of 2% heat-inactivated horse-serum, 0.3 % BSA, 0.05 % fetuin, and 50 µg/ml gentamicin. Media was changed every 2–3 days. After 6 days of differentiation, cells were incubated for 24 hours in DM supplemented with either 0.01% BSA (control) or 100 µM oleate:palmitate (1:1 ratio) bound to 0.01% BSA plus 2 mM carnitine (lipid incubated). Respirometry experiments were performed on day 7 of differentiation. Separate cell aliquots of the same passage number were grown and harvested for analysis for protein content, enzyme activity, and mitochondrial DNA copy number.

RESPIROMETRY EXPERIMENTS

Mature human myotubes were lifted from T-75 culture plates with 0.05% trypsin EDTA, spun for 10 minutes at 1000 rpm, and resuspended in warmed in DMEM (5 mmol/L glucose). An aliquot of cells plus DMEM was reserved for permeabilization reference and data normalization to viable cell count using trypan blue (95-100% viable). No difference in cell number or cell viability was observed between lean and obese or due to the lipid incubation. Myotubes were then spun for an additional 5 minutes at 500 rpm, the supernatant was discarded and cells were resuspended in room temperature respiration buffer (130 mM sucrose, 60 mM potassium gluconate, 3 mM magnesium chloride, 10 mM potassium phosphate, 20 mM HEPES, 0.1% BS; pH 7.4) supplemented with fresh EGTA (1 mM, pH 7.4) and digitonin (7-10 µg/10^6 cells), then

immediately transferred to the respiration chamber (~1-1.5 x 10^6 cells/2 mL). Once oxygen concentration flux stabilized, substrates were added as described (Table 5), allowing for flux stabilization between each addition. Side-by-side experiments of control and lipid incubated cells were performed for each subject. Addition of 10 µM cytochrome C assured that the mitochondrial membrane remained intact. The most stable portion of the oxygen concentration slope was assessed for each condition was normalized to viable cell count.

Specific substrate additions allowed for measurement of state 4 (substrate only, no ADP added), state 3 (substrate + ADP), state 2 (+ oligomycin) and chemically uncoupled (FCCP-stimulated) respiration rates. In addition, various substrates allowed for measurement of lipid or carbohydrate oxidation through Complex I (palmitoyl carnitine + malate {PCM} or pyruvate + malate {PM}) or Complex II (succinate) of the mitochondria. A representative tracing for both control and lipid incubated experiments in cells from a lean donor is shown in Figure 5.

Substrate concentrations were chosen to produce maximal substrate-specific stimulus for both lean and obese. In preliminary experiments we determined that 5 µM palmitoyl carnitine was sufficient to induce maximal increases in respiration and titrations up to 25 µM did not induce additional oxygen consumption. Substrates were prepared at pH 7.4, where necessary, and frozen at -20^{o}C in single use aliquots, except for pyruvate, which was prepared fresh for each experiment day.

PROTEIN ANALYSIS

Cells were lysed and harvested from control and lipid treated flasks using a 4% SDS solution containing protease and phosphatase inhibitors (Sigma; St. Louis, MO). Total protein concentrations were determined by bicinchoninic acid (BCA) protein assay (Pierce; Rockford, IL). Total protein (20 μg) prepared from cell lysates was separated by 7.5% or 12.5% SDS-PAGE, transferred to PVDF membranes (Biorad; Hercules, CA), and then incubated with antibodies diluted in 5% BSA in Tris-buffered saline. Proteins were visualized by horseradish peroxidase conjugated immunoglobulin G. COX-IV and PGC-1α antibodies were purchased from Cell Signaling (Danvers, MA). NRF-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

ENZYME ACTIVITY ANALYSIS

Cells were harvested from control and lipid treated flasks using a 100 mM potassium phosphate solution containing 0.05% BSA. Cells were briefly sonicated and incubated in respective enzyme activity solutions while reading at respective wavelengths in the spectrophotometer. Once a steady rate of enzyme activity was reached, the slope of each sample normalized for a blank control, measurement duration, sample dilution, and appropriate molar extinction coefficients and protein content (BCA protein assay (Pierce; Rockford, IL)). For citrate synthase activity, 20 μL of each sample (approximately 10-15 μg protein) was added to 200 μL of a freshly-

prepared enzyme activity solution (72.5mM Tris, 110 µM DTNB, 1.75 mM oxaloacetate, and 79.5 µM Acetyl CoA; pH 8.3) and read at 412 nm every 50 seconds for ten cycles. Data were normalized to molar DTNB extinction coefficient [((SAMPLE nm/s-CONTROL nm/s)220 µL)/(20 µL*13.6)]. For β-HAD activity, 40 µL of each sample (approximately 20-30 µg protein) and 2 µL 10% Triton were added to 200 µL of a freshly-prepared enzyme activity solution (62.5 µM Tris, 312.5 µM β-nicotinamide adenine, 67.5 µM acetoacetyl CoA, and 2.5 mM EDTA; pH 7.0) and read at 355 nm every 50 seconds for ten cycles. Data were normalized to molar β-NADH extinction coefficient [((SAMPLE nm/s-CONTROL nm/s)240 µL)/(40 µL*6.22)].

MITOCHONDRIAL DNA ANALYSIS

Cells were harvested from control and lipid treated flasks using a 10% SDS solution (pH 8.0) containing proteinase K (Qiagen Inc.; Valencia, CA). Samples were rocked overnight at 50°C to digest all protein. DNA was then isolated using a phenol/chloroform extraction with ethanol precipitation, and resuspended in a 10 mM Tris, 0.1 mM EDTA solution. Total DNA content was determined using Quant-iT PicoGreen reagents (Invitrogen Corp.; Carlsbad, CA). Real-time quantitative PCR (RTQ-PCR) was performed using the ABI PRISM 7400HT Sequence Detection System instrument and software with Taqman® Universal PCR Master Mix in accordance with manufacturer's instructions (Applied Biosystems, Inc. (ABI); Foster City, CA). Relative mitochondrial DNA content (Cyt b) was determined using the number of cycles

necessary to reach threshold (Ct). All samples were run on the same plate and values normalized to genomic DNA (β-globin). A standard curve consisting of a serially diluted pool of aliquots from each of the samples was also analyzed to assure assay efficiency. Primer and probe sequences are listed in Table 6.

Statistical Analyses

T-tests were used to compare control and lipid incubated conditions and differences between cells from lean and obese donors. Statistical significance was denoted at $P < 0.05$ level and data are presented as the mean ± SEM.

Results

Participant Characteristics

Participant characteristics are listed in Table 4. By design, the obese participants were heavier and had greater BMIs than the leans. Blood glucose and triglyceride values were not different between the groups, although insulin levels and HOMA values were higher in the obese.

Markers of Mitochondrial Content/Activity

Mitochondrial DNA copy number was not different between cells from lean and obese at baseline (513 ± 17 and 489 ± 75 AU in lean and obese, respectively; lean n =7, obese n=5). The 24 hour lipid incubation increased relative mitochondrial DNA copy

number in the cells from lean donors and tended to decrease copy number in the cells from obese donors (+16%, $P < 0.05$, and -13%, $P = 0.06$, in lean and obese, respectively; Figure 9). Corroborating these data was a body size x lipid incubation interaction for COX-IV protein content ($P < 0.05$; lean n=6, obese n=6), where the change from control to lipid incubation tended to be difference between cells from lean and obese donors. $P = 0.07$; Figure 9). Citrate synthase activity was not different between cells from lean and obese at baseline, nor was there any effect of lipid incubation (lean n=7, obese n=5). β-HAD activity was not different between lean and obese at baseline, but decreased following lipid incubation when both lean and obese were combined (0.23 ± 0.03 and 0.18 ± 0.02 $\mu M \bullet \mu g\ protein^{-1} \bullet min^{-1}$ for control and lipid incubated, respectively; $P < 0.05$; lean + obese n=12).

PGC-1α and NRF-1 protein content were not different between cells from lean and obese donors and were unchanged as a result of lipid incubation (Figure 10), indicating no observable difference in mitochondrial biogenesis.

RESPIROMETRY

Respiration was not different between cells cultured from lean and obese donors in any respiration state (basal state 4, ADP-stimulated state 3, state 3 through mitochondrial complex II, or oligomycin or FCCP supported respiration) in the presence of either palmitoyl carnitine + malate (PCM) or pyruvate + malate (PM) in either control

or lipid-incubated cells. However, ADP-stimulated respiration tended to be higher in cells cultured from lean donors in the presence of PCM (P = 0.07; Figure 6)

Following the 24 h lipid incubation, myotubes from lean, but not obese donors exhibited increased state 3 mitochondrial respiration relative to the control cells in the presence of both PCM and PM (+95% and +39% for PCM and PM, respectively; P < 0.05, Figure 6). ADP-stimulated respiration through Complex II (+ succinate + glutamate) was elevated in the leans following the 24 h lipid incubation in the presence of PM (+23%; *P* < 0.05) but not PCM (+ 7%; n.s., Figure 7) experiments. Uncoupled respiration was also increased in the leans following lipid incubation (+29% and +26% for PCM and PM, respectively; *P* < 0.05) but unchanged in the obese (+7% and +9% for PCM and PM, respectively; n.s., Figure 7). State 2 respiration remained unchanged with lipid incubation and was similar between groups.

To account for differences in mitochondrial content, respiration data were also normalized to mitochondrial DNA copy number. When normalized to mitochondrial DNA, state 3 respiration rates were elevated following lipid incubation in cells from lean donors both in the presence of PCM and PM (+28% and +25% for PCM and PM, respectively; *P* < 0.05, Figure 8), as was observed when the data were normalized to cell count. Similar results were observed for uncoupled respiration rates, normalized to mitochondrial DNA copy number (+17% and +15% for PCM and PM, respectively; *P* < 0.05, Figure 8). However, the 24 h lipid incubation did not affect respiration rates in the

cells from obese donors in the presence of either PCM or PM (Figure 8), similar to the when data normalized to cell count. These data indicate that changes in mitochondrial DNA copy number were not solely responsible for lipid incubation-induced changes in mitochondrial oxidation that were observed in the cells from lean donors.

DISCUSSION

Skeletal muscle oxidative capacity is impaired in obese and insulin resistant populations, though it is unclear whether this is attributable to impaired mitochondrial function (Kelley et al., 2002; Mogensen et al., 2007; Phielix et al., 2008; Ritov et al., 2005), reduced mitochondrial content (Boushel et al., 2007; Holloway et al., 2006; Holloway et al., 2007), or both (for review, see (Holloway et al., 2009)). Using a unique method of measuring mitochondrial respiration in cultured myotubes from lean and obese human donors, we have identified an obesity related mitochondrial dysfunction that is evident as an inability to upregulate metabolism (metabolic inflexibility) in response to a lipid challenge (24 h lipid incubation). While cells cultured from our lean donors exhibited large increases in state 3 and uncoupled mitochondrial respiration following a 24-hour mixed lipid incubation (Figure 7), we did not observe a similar response in the cells from obese donors. These findings suggest that phenotypic skeletal muscle metabolic inflexibility evident in obese humans is preserved in this cell culture model.

Metabolic inflexibility in obese and type 2 diabetics has been described as an impaired ability to suppress lipid oxidation in response to insulin-stimulation (for review, see (Kelley et al., 1999)). Lean individuals also respond to excess dietary fat by increasing whole body lipid oxidation (Cameron-Smith et al., 2003; Chokkalingam et al., 2007; Pehleman et al., 2005; Peters et al., 2001), though obese and previously obese individuals do not (Astrup et al., 1994; Thomas et al., 1992). A novel finding of the present study is that this same inability to respond to a lipid challenge is retained in primary cell cultures from obese donors (Figure 6). This observation not only implies an innate characteristic preserved in skeletal muscle satellite cells, but also establishes a new model in which to study the respiratory defects observed in obese humans. By using a culture model one can strictly control the extracellular environment to examine skeletal muscle metabolism without interference from alternate organs and the circulating hormonal and metabolic milieu.

Myotubes from lean donors exhibited elevated State 3 and uncoupled respiration following lipid exposure in the presence of both palmitate (PCM) and pyruvate (PM) through Complex I and Complex II (+ succinate). Because these effects were present regardless of substrate or respiration state, it can be hypothesized that there were increases in total mitochondrial capacity, as a result of the 24 h lipid incubation, rather than one specific enzyme or pathway. In support, we observed elevated mitochondrial DNA copy number in the lean subjects with the 24 h lipid

incubation (Figure 9). In contrast, myotubes from obese donors did not exhibit similar increases in mitochondrial respiration (metabolic *inflexibility*) regardless of decreases in mitochondrial DNA content. These data indicate that specific mitochondrial dysfunction is evident in obesity, particularly in the inability of skeletal muscle to respond to a lipid substrate challenge.

In a previous report, 20 hours of lipid incubation (8mM oleate), induced PGC-1α gene expression and increased mitochondrial activity in culture myotubes from human donors (Staiger et al., 2005). Therefore, despite our much lower oleate incubation (50 μM), we were not surprised to observe slight, though significant increases in mitochondrial content in cells cultured from our lean donors. However, a novel finding of the present study was mitochondrial content *decreased* in cells from obese donors (Figure 9). In the current investigation, changes in PGC-1α or NRF-1 protein content did not account for changes in mitochondrial content, though these data do not negate potential differences in post-translational modifications and activation that could contribute to differences in mitochondrial biogenesis. Further investigations into potential defects in lipid-induced mitochondrial biogenesis and/or mitoptosis in skeletal muscle of obese individuals are warranted.

A lower State 3 respiration has been reported in permeabilized skeletal muscle fibers of type 2 diabetics compared with non-diabetic, weight-matched controls in the presence of PM (Mogensen et al., 2007) and malate + glutamate + succinate (Phielix et

al., 2008), but not PCM (Mogensen et al., 2007; Phielix et al., 2008), even when normalized to mitochondrial content. However, Boushel et al. (2007) reported that differences in State 3 (glutamate + malate) respiration in type 2 diabetics and weight-matched cohorts were eliminated when respiration was normalized to mitochondrial DNA copy number. In the control condition, we did not observed differences in state 3 respiration between cells from lean and obese (Figure 6), nor differences in mitochondrial content in the non-lipid incubated cells (Figure 9). The present investigation agrees that in an unstimulated condition (control cells) mitochondrial content may be a good indicator of respiration rates. However, once stimulated with the 24 h lipid incubation there is evidence of reduced oxidative capacity in cells from obese donors that is not explained by mitochondrial content, indicating metabolic inflexibility.

One prior investigation has reported that myotubes cultured from type 2 diabetics *are* metabolically flexible in response to acute glucose and palmitate oxidation, where there is evidence of Reverse Randle-Cycle activity (Gaster, 2007). This appears to be at least partially regulated through CPT1, given that etomoxir reduced glucose-dependent inhibition of palmitate oxidation (Gaster, 2007). However, these experiments (Gaster, 2007) only investigated the acute regulation of palmitate oxidation and do not address more prolonged exposure to lipids that can lead to lipid accumulation within the cell (Aas et al., 2006; Wensaas et al., 2009) and potentially

regulate (or *dis*-regulate) metabolism. Elevated dietary lipid intake increases skeletal muscle lipid content in both humans (Schrauwen-Hinderling et al., 2005) and rodents (Koves et al., 2008) and is reported to exacerbate phenotypic differences in lipid metabolism between lean and obese cohorts (Koves et al., 2008). With longer lipid incubations (4 days), myotubes cultured from type 2 diabetics exhibited a less robust increase in $^{14}CO_2$ production from labeled palmitate incubation, than was observed in cells from weight-matched counterparts (20% lower). However, these measurements were performed in the non-ADP stimulated state, so it is unclear if this lipid exposure affected mitochondrial oxidative capacity. The present investigation identifies these phenotypic differences in cultured myotubes from lean and obese humans in the disparate responses to controlled lipid incubation in both state 3 and chemically uncoupled respiration and recognizes a distinct mitochondrial dysfunction not entirely explained by differences in mitochondrial content.

In conclusion, we show here that metabolic inflexibility is preserved in cultured myotubes from obese human donors and that this impairment is evident at the level of mitochondrial respiration. In addition, it appears that the inability to respond to lipid incubation is not entirely accounted for by diminished mitochondrial content. The skeletal muscle of obese individuals exhibits an impaired ability to respond to a metabolic challenge, namely, excess lipid. This is the first report of such findings and

introduces a novel model for measuring mitochondrial dysfunction in skeletal muscle with obesity.

TABLE 4. PARTICIPANT CHARACTERISTICS.

Data presented for age, stature, body mass, BMI, plasma glucose, plasma insulin, HOMA, serum cholesterol, and serum triglycerides in lean and obese participants. All data are presented as mean ± SEM. *Indicates significant difference between the groups ($P < 0.05$).

	Lean (n=7)	Obese (n=7)
Age (y)	22.3 ± 0.9	21.8 ± 1.1
Stature (cm)	181.6 ± 2.8	182.8 ± 2.0
Mass (kg)	71.7 ± 3.1	130.2 ± 7.9*
BMI (kg/m^2)	21.7 ± 0.8	39.0 ± 2.0*
Glucose (mmol/L)	4.93 ± 0.21	4.96 ± 0.15
Insulin (µIU/L)	5.3 ± 1.1	15.9 ± 1.4*
HOMA	1.2 ± 0.3	3.6 ± 0.3*
Cholesterol (mg/dL)	176.7 ± 9.0	168.5 ± 18.6
Triglycerides (mg/dL)	147.9 ± 36.8	137.2 ± 35.8

TABLE 5. RESPIROMETRY PROTOCOL FOR HSMC.

Substrate additions for respirometry experiments are described below. Two experiments were performed for each set of cells (control and 24 hour lipid incubated) including respiration in the presence of PCM or PM.

	Substrate		Concentration
In Suspension	Digitonin	**Dig**	7-10 µg/10^6 cells
1	Pyruvate	**PM**	2 mM Pyruvate
	Malate		1 mM Malate
OR			
1	Palmitoyl Carnitine	**PCM**	5 µM Palmitoyl Carnitine
	Malate		1 mM Malate
2	ADP	**D**	2 mM
3	Cytochrome C	**CytC**	10 µM
4	Succinate	**S**	3 mM
5	Glutamate	**G**	2 mM
6	Oligomycin	**O**	2.5 µg/mL
7	FCCP	**F**	2 µM

TABLE 6. PRIMER AND PROBE SEQUENCES.

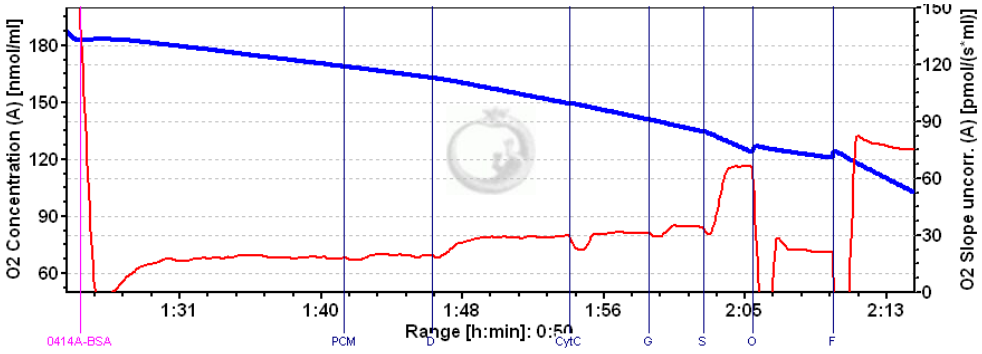
Primer and Probe sequences used to determine mitochondrial DNA copy number analyses using RT-PCR for and β-globin (genomic DNA) and Cyt b (mitochondrial DNA). Sequences are listed in 5’ to 3’ orientation.

	Forward Primer	Reverse Primer	Probe
β-Globin	TGAAGGCTCATGGCAAGAAA	AAAGGTGCCCTTGAGGTTGTC	CCAGGCCATCACTAAAGGCACCGA
Cyt*b*	GACGCCTCAACCGCCTTT	GCGGATGATTCAGCCATAATTTA	CATCAATCGCCCACATCACTCGAGAC

FIGURE 5. REPRESENTATIVE OXYGRAPH TRACING OF HSMC FROM ONE LEAN DONOR.

Respiration of permeabilized HSMC in the presence of various substrates as listed in Table 5 following incubation with either BSA alone (control, Panel A) or BSA + 100 µM oleate:palmitate [1:1] (O:P, Panel B). The blue line represents the oxygen concentration within the respiration chamber, while the red line represents the oxygen flux (JO_2), or the slope of the blue line.

A


O2 Concentration (A) [nmol/ml]
O2 Slope uncorr. (A) [pmol/(s*ml)]
180
150
120
90
60
150
120
90
60
30
0
1:31
1:40
1:48
1:56
2:05
2:13
Range [h:min]: 0:50
0414A-BSA
PCM
D
CytC
G
S
O
F

B

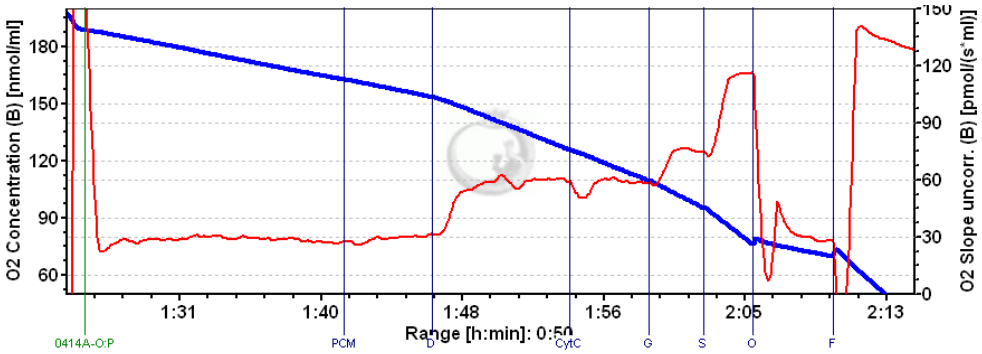

O2 Concentration (B) [nmol/ml]
O2 Slope uncorr. (B) [pmol/(s*ml)]
180
150
120
90
60
150
120
90
60
30
0
1:31
1:40
1:48
1:56
2:05
2:13
Range [h:min]: 0:50
0414A-O:P
PCM
D
CytC
G
S
O
F

FIGURE 6. MITOCHONDRIAL RESPIRATION IN MYOTUBES FROM LEAN AND OBESE DONORS.

State 4 and State 3 respiration rates in cultured myotubes from lean (open bars) and obese (filled bars) donors in the presence of PCM (Panel A) and PM (Panel B) with and without 24 hour lipid incubation. Data are presented as the mean ± SEM of the oxygen flux per second in 2 mL chamber volume, normalized to total cell count. * Indicates significant response to the lipid incubation.

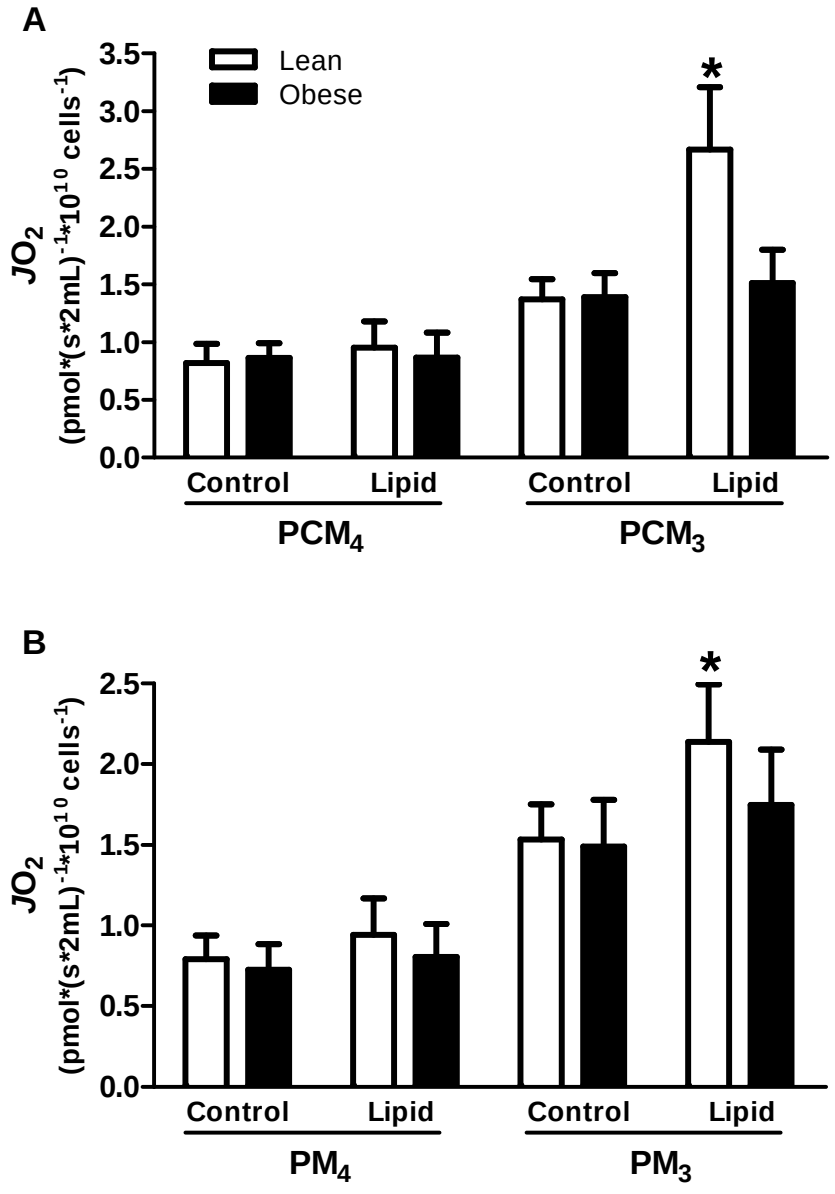

A
Lean
Obese
JO_2 (pmol*(s*2mL)$^{-1}$*10^{10} cells^{-1})
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Lipid
Control
Lipid
PCM_4
PCM_3
B
JO_2 (pmol*(s*2mL)$^{-1}$*10^{10} cells^{-1})
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Lipid
Control
Lipid
PM_4
PM_3

FIGURE 7. UNCOUPLED RESPIRATION IN MYOTUBES FROM LEAN AND OBESE DONORS.

State 3 (+ glutamate + succinate) and uncoupled respiration (+ FCCP) rates in cultured myotubes from lean (open bars) and obese (filled bars) donors in the presence of PCM (Panel A) and PM (Panel B) with and without 24 hour lipid incubation. Data are presented as the mean ± SEM of the oxygen flux per second in 2 mL chamber volume, normalized to total cell count. * Indicates significant response to the lipid incubation.

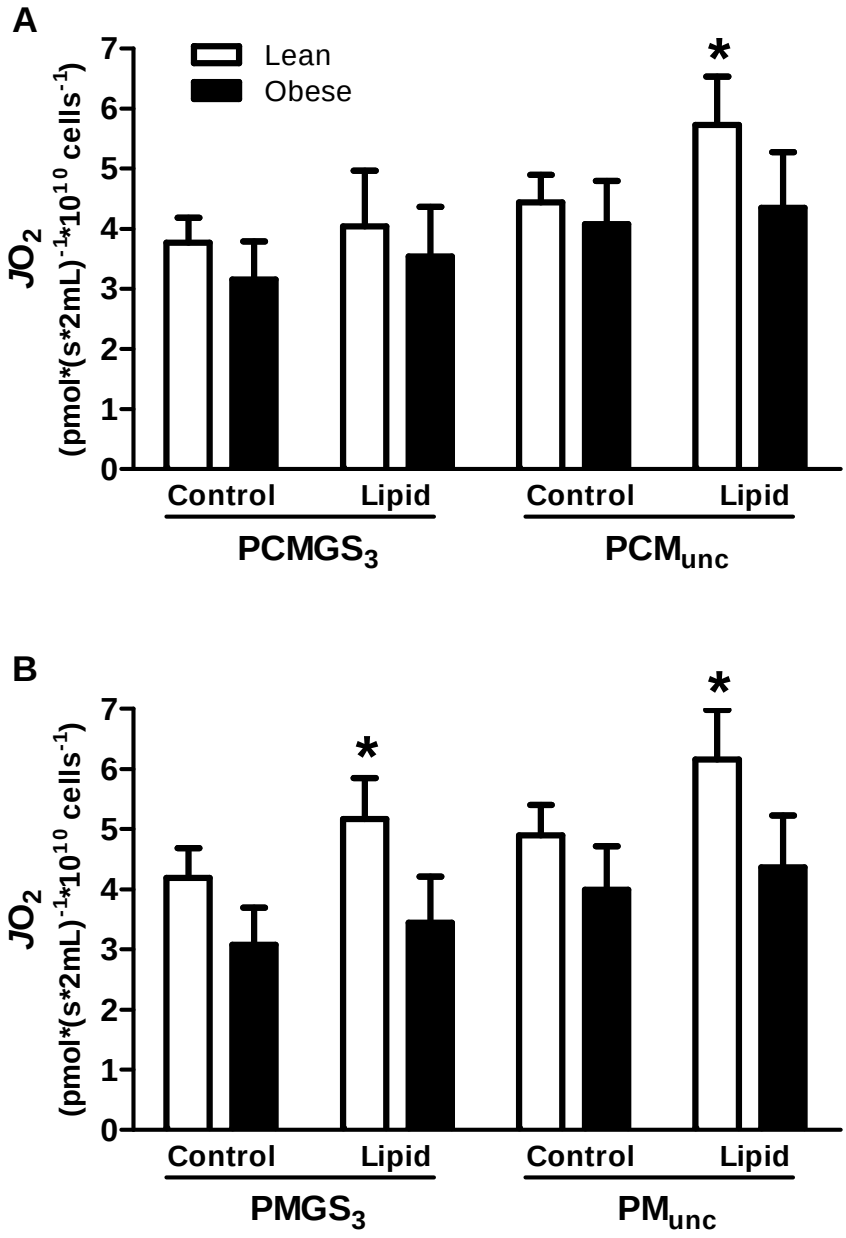

A
Lean
Obese
JO_2
(pmol*(s*2mL)$^{-1}$*10^{10} cells^{-1})
0
1
2
3
4
5
6
7
*
Control
Lipid
Control
Lipid
$PCMGS_3$
PCM_{unc}
B
JO_2
(pmol*(s*2mL)$^{-1}$*10^{10} cells^{-1})
0
1
2
3
4
5
6
7
*
*
Control
Lipid
Control
Lipid
$PMGS_3$
PM_{unc}

FIGURE 8. MITOCHONDRIAL RESPIRATION IN CONTROL AND LIPID INCUBATED HSMC.

State 3 and uncoupled respiration in control and lipid-incubated myotubes cultured from lean (open bars) and obese (filled bars) donors in the presence of PCM (Panel A) and PM (Panel B). Data are presented as the mean ± SEM of the oxygen flux per second in 2 mL chamber volume, normalized to mitochondrial DNA copy number and total cell count. * Indicates significant response to the lipid incubation.

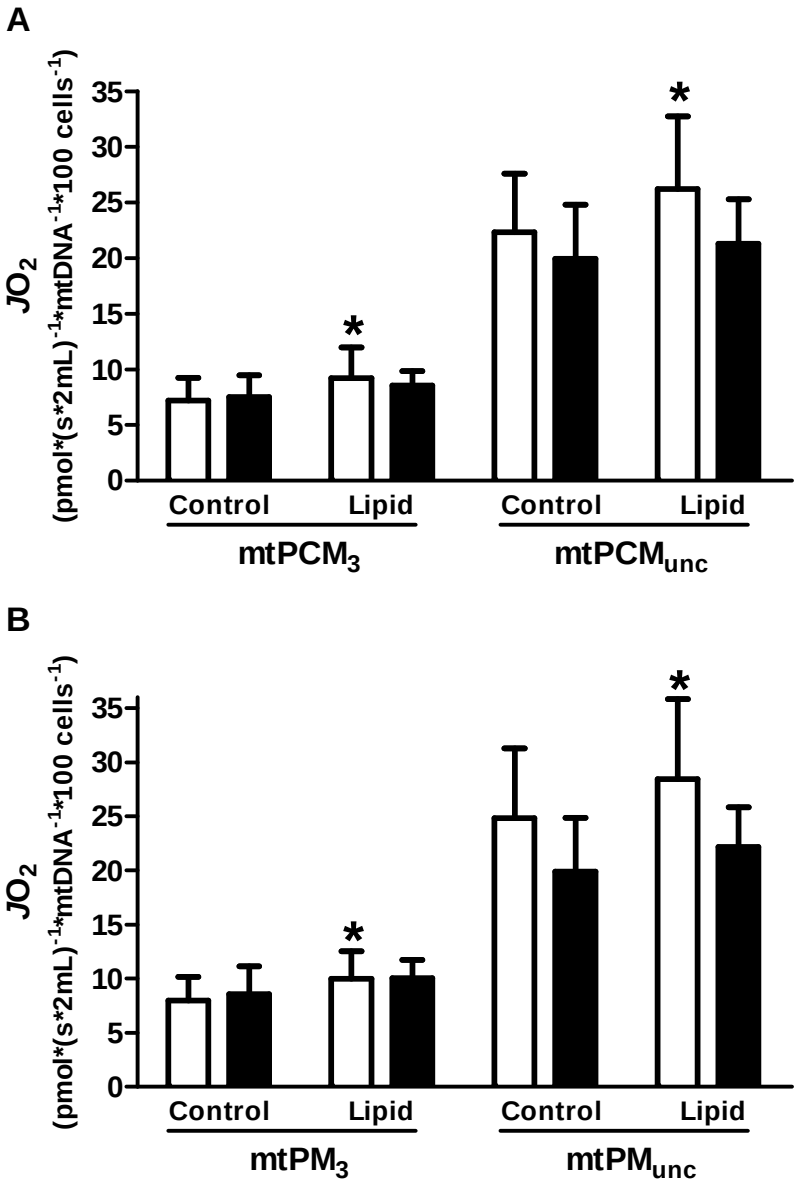

A
JO_2
(pmol*(s*2mL)$^{-1}$*mtDNA^{-1}*100 cells^{-1})
35
30
25
20
15
10
5
0
*
*
Control
Lipid
Control
Lipid
mtPCM$_3$
mtPCM$_{unc}$
B
JO_2
(pmol*(s*2mL)$^{-1}$*mtDNA^{-1}*100 cells^{-1})
35
30
25
20
15
10
5
0
*
*
Control
Lipid
Control
Lipid
mtPM$_3$
mtPM$_{unc}$

FIGURE 9. MARKERS OF MITOCHONDRIAL CONTENT WITH LIPID EXPOSURE IN HSMC.

Mitochondrial DNA copy number (Panel A) and COX-IV protein (Panel B) with and without 24 hour lipid incubation in cultured myotubes from lean (open bars) and obese (filled bars) donors. All values are expressed as mean ± SEM. * Indicates significant response to the lipid incubation. # indicates significant differences from lean. Significance is indicated at $P < 0.05$.

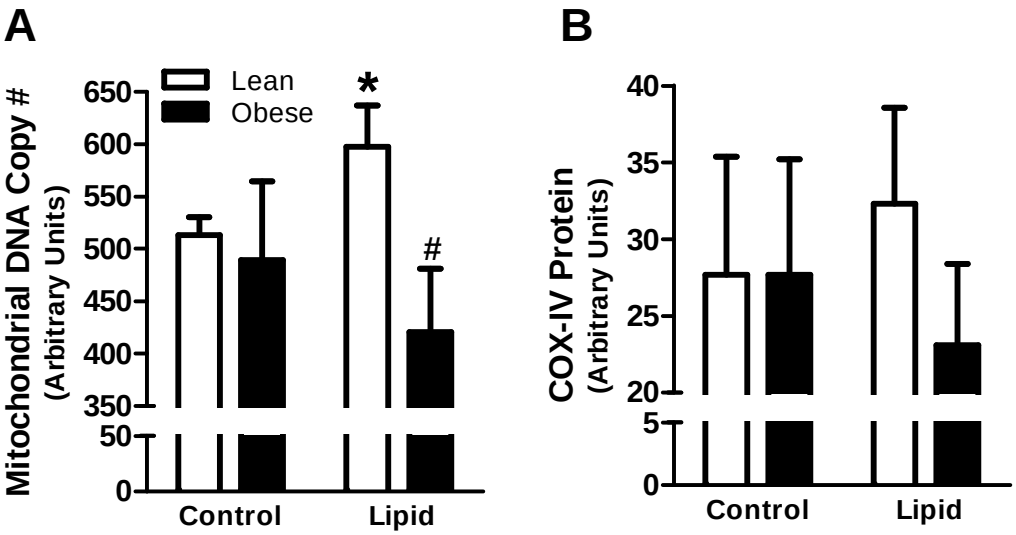
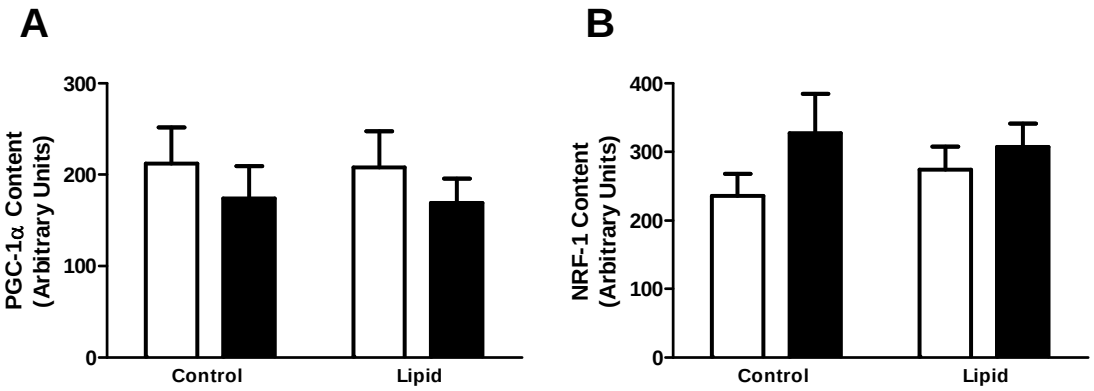

A
Mitochondrial DNA Copy #
(Arbitrary Units)
650
600
550
500
450
400
350
50
0
Lean
Obese
*
#
Control
Lipid
B
COX-IV Protein
(Arbitrary Units)
40
35
30
25
20
5
0
Control
Lipid

FIGURE 10. PGC-1α AND NRF-1 PROTEIN CONTENT WITH LIPID EXPOSURE IN HSMC.

PGC-1α (Panel A) and NRF-1 protein (Panel B) with and without a 24 hour lipid incubation in cultured myotubes from lean (open bars) and obese (filled bars) donors. Value means are expressed arbitrary units ± SEM.


A
PGC-1α Content
(Arbitrary Units)
300
200
100
0
Control
Lipid
B
NRF-1 Content
(Arbitrary Units)
400
300
200
100
0
Control
Lipid

CHAPTER 4: INTEGRATED DISCUSSION

Skeletal muscle of obese individuals has a reduced capacity to oxidize lipids and some data suggests an inability to respond to a metabolic stimulus is present in these individuals, particularly in relation to increased carbohydrate oxidation in face of insulin stimulation (Kelley et al., 1999). Some data also suggests a similar 'metabolic inflexibility' with respect to lipid oxidation in response to lipid exposure obese individuals (Astrup et al., 1994). This inability to respond to a metabolic stimulus has been linked with both obesity and insulin resistance and the nature of this phenomenon may provide valuable insight into these co-morbidities. The ability to regulate lipid oxidation in response to lipid exposure is impaired in the skeletal muscle of obese individuals, and is evident with both 5 days of high fat feeding in obese humans, as well as with 24 hours of lipid incubation in cultured myotubes from obese human donors.

Chapter 2 focused on the effects of lipid exposure on skeletal muscle mRNA content in the form of a 5 day, 65% fat diet in lean and obese humans. Whereas skeletal muscle from lean individuals exhibited increased mRNA content of many genes associated with lipid oxidation, skeletal muscle from obese individuals did not. Likewise, whereas skeletal muscle from lean individuals showed reduced long and medium chain lipid species from Pre- to Post-HFD, the skeletal muscle from the lean participants only displayed reduced content of long chain lipid species, with no change in medium chain lipids, and even increased lipid content in some cases. These data suggest that there

may be an impairment in lipid oxidation downstream of MCAD in skeletal muscle of obese individuals in response to high fat feeding, or perhaps there is reduced TCA cycle activity, as has been presented in other previously in other models of obesity (Koves et al., 2008). In retrospect, measuring explicit skeletal muscle lipid oxidation may have presented a more physiologically relevant outcome variable, though limitations in tissue collection precluded these measurements consistently. Nonetheless, many have previously shown that changes in skeletal muscle PDK4 mRNA content and activity are associated with concomitant increases in lipid oxidation, particularly with regard to high fat feeding in lean humans (Cameron-Smith et al., 2003; Peters et al., 2001), and our most robust responses were with PDK4 mRNA content, lending more physiological significance to our mRNA data.

In order to better determine the nature of this 'metabolic inflexibility' in skeletal muscle of obese humans, Chapter 3 examines the effect of lipid exposure on skeletal muscle mitochondrial function in a unique model of permeabilized cultured myotubes harvested from lean and obese human donors. This method allowed for explicit measurement of various aspects of mitochondrial respiration in a model of skeletal muscle that is not influenced by other organs or the physiologic metabolic milieu. In doing so, we observed similar 'metabolic inflexibility' in response to lipid exposure in these myotubes cultured from obese humans as was observed in the skeletal muscle on obese humans in response to high fat feeding. This inflexibility was observed at many

levels of mitochondrial respiration, as well as in the ability to induce mitochondrial biogenesis. These data indicate that this 'metabolic inflexibility' is present in overall mitochondrial function. Because mitochondrial biogenesis appears to be an important aspect of this metabolic inflexibility and many of the metabolic regulators for lipid oxidation are also regulators for mitochondrial biogenesis (i.e PGC-1α), perhaps further research in the control of this adaptation may be warranted.

In obese humans, metabolic control of lipid oxidation in skeletal muscle is impaired at the level of mRNA content, and is evident for many genes involved in this mechanism. In addition, in myotubes from obese donors, overall mitochondrial function was impaired in response to lipid exposure, at many levels of mitochondrial respiration, as well as mitochondrial biogenesis. Taken together, these data suggest that there is a global inability to respond to lipid exposure in skeletal muscle of obese individuals. In addition, the fact that this inability to respond to lipid exposure was observed in satellite cells cultured from human donors, suggests a genetic or epigenetic component. Further research into the mechanism of this global impairment in the ability to respond to a metabolic stimulus may provide valuable insight into potential treatment options for obesity, or perhaps a mechanism whereby obesity is exacerbated by repeated metabolic overloads to which the skeletal muscle is unable to respond.

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APPENDIX A: DETAILED METHODS

CONTROLLED HIGH-FAT DIET

Subjects consumed their normal standard mixed diet (55% carbohydrate, 15% protein, 30% fat) diet prior to the experiment. The HFD was designed individuals for each participant and contained 60-65% of energy from fat, 15% of energy from protein, and 20-25% of energy from carbohydrate. Daily caloric requirements were based on body size using the Harris-Benedict equation (1928) with ideal body weight (IBW) adjustments made for obese participants (Males: 48.2 kg + 2.7 kg every inch over 5 ft. of stature, Females: 45.5 kg + 2.3 kg every inch over 5 ft. of stature); American Dietetic Association Nutrition Care Manual). The high-fat meal on days 1 and 6 was also designed to contain 60-65% of energy from fat and will comprise 35% of each subject's daily energy intake. All meals consisted of pre-measured snack foods and prepared frozen meals, which were provided to the participants. Subjects were asked to adhere to the planned menu, to maintain their normal physical activity levels, and to refrain from alcohol consumption for the duration of the study. All diet records, meals, and the high fat diets were analyzed using Nutritionist Pro^{TM} Nutrition Analysis Software (Axxya Systems) to assure proper energy intake and macronutrient composition. Food diaries were maintained by the subjects during the HFD as an

indicator of compliance. A timeline of dietary intervention, muscle biopsy, and blood samplings is presented in Figure 11.

RNA Extraction/Quantification

Skeletal muscle were obtained from the vastus lateralis at 4 time points throughout the experiment for the analysis of mRNA and protein content. Total RNA was isolated from ~30 mg of frozen muscle using either the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) with on-column DNase digestion using the RNase-Free DNase Set (QIAGEN, Inc.) to remove residual DNA or a guanidine thiocyanate digestion (100mM guanidine thiocyanate, 20mM sodium acetate, 0.5% n-lauryl-sarcosine) with phenol extraction method and an additional lithium chloride (4M) step to solubolize residual DNA. RNA was extracted using the same method for all samples of each subject. Total RNA was reverse transcribed into cDNA using the Superscript III Reverse Transcriptase protocol (Invitrogen Corp., Carlsbad, CA) and was quantified in triplicate with Quant-iT PicoGreen reagents (Invitrogen Corp., Carlsbad, CA).

DNA Extraction/Mitochondrial DNA Quantification

Differentiated cultured muscle cells from lean and obese individuals with or without 24h 100 µM oleate:palmitate (1:1 ratio) incubation were harvested for the analysis of mitochondrial copy number per total nuclear DNA content.

Total DNA was isolated from cells following overnight digestion at 50°C in buffer containing Proteinase K (100mM NaCl, 10mM Tris, 25mM EDTA, 0.25% SDS, 0.1 mg/mL proteinase K; pH 8) using a phenol/chloroform extraction method. Total DNA and mtDNA was measured using quantitative RT-PCR with β-globin and cytochrome *b* (Cyt*b*) primer/probe sets, respectively (Table 6).

QUANTITATIVE RT-PCR

Real-time quantitative PCR (RTQ-PCR) was performed using the ABI PRISM 7400HT Sequence Detection System instrument and software with Taqman® Universal PCR Master Mix in accordance with manufacturer's instructions (Applied Biosystems, Inc. (ABI), Foster City, CA). Relative gene expression levels were determined using the number of cycles necessary to reach threshold (Ct). The Ct values from RTQ-PCR will then be compared with a standard curve consisting of a serially diluted pool from each of the samples. All samples were run on the same plate and values normalized to the amount of cDNA that was originally added to each reaction well. Primer and probe sequences for all genes assessed are presented in Table 2. Endogenous control gene expression (18S) were also measured and compared with each gene of interest to assure assay efficiency.

Protein Extraction

Total protein was extracted from either muscle tissue or harvested cell culture using a standard lysis buffer (20mM Tris-HCL, 10mM sodium fluoride, 1mM EDTA, 4% SDS, 20% glycerol; pH 6.8) containing phosphatase and protease inhibitors. Samples with be homogenized or sonicated, respectively. In the case of muscle homogenate, particulates were spun down and protein content in the remaining suspension was measured using the standard BCA assay protocol (Pierce; Rockford, IL).

Western Blot

Proteins from total cell or tissue lysates were separated out by 10% SDS-PAGE, then transferred to PVDF membranes and blocked with diluted, reconstituted dry-milk-powder (5%). Membranes were then incubated with antibodies for proteins of interest followed by horseradish peroxidase conjugated antibodies with species specific recognition of primary antibodies. Proteins were then visualized using a chemiluminescence assay.

Skeletal Muscle Homogenate Palmitate Oxidation

Muscle palmitate oxidation was measured as previously described (Kim et al., 2000). Approximately 50–60 mg of tissue was collected in 200 µL of a modified sucrose-EDTA medium containing 250 mM sucrose, 1 mM EDTA, 2mM

ATP and 10 mM Tris·HCl, pH 7.4. Samples were minced thoroughly with scissors and then diluted 20-fold with additional sucrose-EDTA buffer. Tissue was placed on ice and homogenized with a Teflon pestle on glass for 30 s. Muscle homogenate (40 µL) was added to incubation wells in a sealed, modified, 48-well plate with a channel cut between the adjacent trap wells, which contained 200 µL of 1 N sodium hydroxide for the collection of liberated $^{14}CO_2$. Incubation buffer {final concentrations: 0.2 mM palmitate ([1-14C]palmitate at 0.5 µCi/mL), 62.5 mM sucrose, 10 mM Tris·HCl, 12.5 mM potassium phosphate, 100 mM potassium chloride, 0.1 mM malate, 2 mM ATP, 1 mM dithiothreitol, 0.1 mM β-NAD, 1 mM L-carnitine, 0.05 mM coenzyme A, and 0.5% fatty acid-free bovine serum albumin, 160 µL, pH 7.4} was added to the wells to initiate the reaction. Following 30 min of incubation at 37°C, 100 µL of 70% perchloric acid was added to terminate the reaction. The trap wells were sampled for label incorporation into $^{14}CO_2$, which was determined by scintillation counting using 4 mL of Uniscint BD (National Diagnostics, Atlanta, GA). In addition to complete oxidative products ($^{14}CO_2$), incomplete oxidative products [acid-soluble metabolites (ASM)] were also measured as described previously (Kim et al., 2000). The ratio of incomplete (ASM) to complete ($^{14}CO_2$) radiolabeled products was determined to provide an index of incomplete to complete FAO. Results are presented in Figure 15.

PRIMARY CULTURE OF HUMAN SKELETAL MUSCLE CELLS

Muscle samples weight 50-100 mg from *vastus lateralis* needle biopsy was used for cell culture. Tissue was immediately transferred to ice-cold DMEM and cleaned free from adipose and connective tissues in Hank's balanced salt solution. Tissue was incubated with trypsin cocktail (0.25% trypsin, 0.1% type IV collagenase, 0.1% BSA) for 30 minutes in shaker bath at 37°C to isolate satellite cells. Cells were re-suspended in growth media (low glucose DMEM containing 10% FBS) and pre-plated onto uncoated T-25 tissue culture flasks for 1-3 hours in order to remove fibroblasts. Cells were then transferred to Type I collagen-coated T-25 flask for attachment and growth. Cells were cultured at 37°C in a humidified incubator with 5% CO_2. When cells (myoblasts) reached 70% confluence, they were transferred to Type I collagen-coated T-75 culture flasks. When cells reached 80-90% confluence, differentiation was induced using differentiation media (low glucose DMEM containing 2% heat-inactivated horse-serum). Cell culture experiments were carried out on mature myotubes on day 7 of differentiation.

WHOLE CELL RESPIROMETRY

On day 6 of differentiation, cultured myotubes from lean and obese humans were incubated in differentiation media supplemented with either 0.1% BSA or 50µM oleate + 50µM palmitate + 0.1% BSA + 200 mM carnitnine for 24h.

Immediately prior to the experiments (day 7) cells were lifted from the culture flasks with a 0.05% trypsin EDTA solution and resuspended in low glucose DMEM. Cell count and viability were measured from these samples, using trypan blue to assess cell viability. For permeabilization, cells were spun down and resuspended in 2 mL of an room temperature sucrose-based respiration buffer containing 7-10 µg of digitonin per million cells. Permeabilized cells and buffer were transferred to the respiration chamber and oxygen flux was measured using Oroboros ® DatLab Software.

For whole cell respiration, aliquots were reserved for normalization to protein content and remaining cells were transferred to the respiration chamber and oxygen consumption was measured in the presence of 5 µg/mL of oligomycin followed by titrated amounts of FCCP up to 24 µM. Baseline respiration measurements are presented in Figure 17.

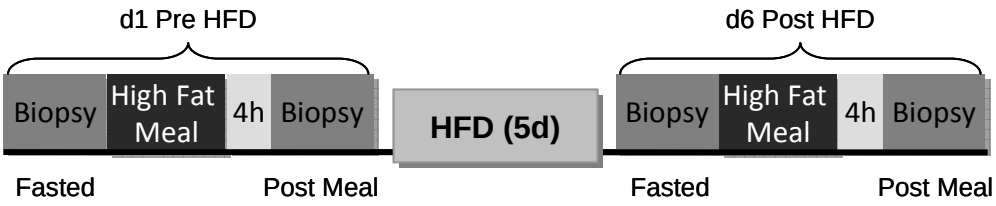
CONFOCAL MICROSCOPY

Cultured human myoblasts from lean and obese humans will be plated and differentiated into collagen coated, glass bottom petri-dishes. On day 5 of differentiation cells will be incubated in low glucose DMEM containing either 0.5% BSA or 500µM oleate + 0.5% BSA for 24h. Thirty minutes prior to the experiments (day 6), 100 µM MitoTracker Red® (Invitrogen Corp.) was added to

the dishes. After 30 minutes, cells will be washed in PBS and mitochondria content and morphology will be visualized using confocal technology Figure 16.

FIGURE 11. CONTROLLED HIGH FAT DIET PROTOCOL

The HFD feeding and sampling protocol. Before (day 1) and after (day 6) of the 5 day HFD, biopsies and blood draws were performed in the Fasted and 4h Post Meal conditions.


d1 Pre HFD
d6 Post HFD
Biopsy
High Fat Meal
4h
Biopsy
HFD (5d)
Biopsy
High Fat Meal
4h
Biopsy
Fasted
Post Meal
Fasted
Post Meal

APPENDIX B: ADDITIONAL RESULTS – CHAPTER 2

FIGURE 12. FASTING PLASMA B-HYDROXYBUTYRATE PRE- AND POST-HFD.

Data are fasting plasma β-hydroxybutyrate levels in the Pre- and Post-HFD conditions in lean and obese individuals. All values are expressed as mean ± SEM. * Indicates a significant increase in β-hydroxybutyrate in both groups combined ($P < 0.05$).

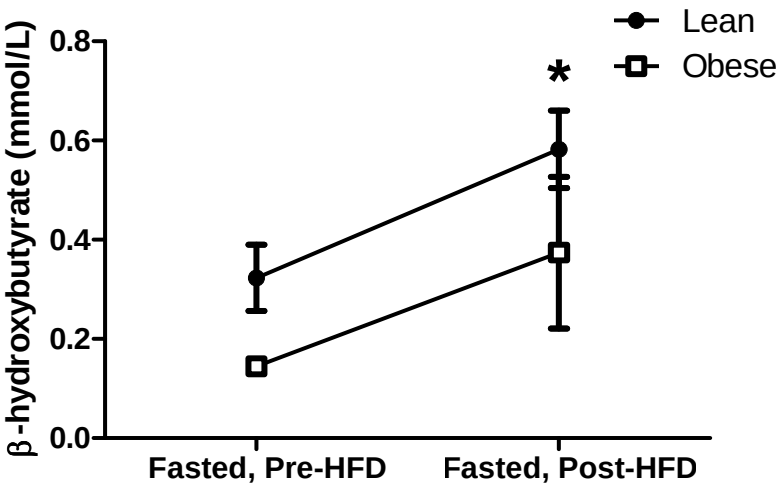

Lean
Obese
β-hydroxybutyrate (mmol/L)
0.8
0.6
0.4
0.2
0.0
*
Fasted, Pre-HFD
Fasted, Post-HFD

FIGURE 13. EFFECT OF HFD ON PDK4 MRNA CONTENT AND PDH PHOSPHORYLATION.

Value means are expressed as the fold change from the Pre- to Post-HFD conditions where the Pre-HFD condition is set at 1 (indicated by the dashed line). Changes in PDK4 mRNA content exhibited significant overall interaction for HFD between lean and obese (Panel A), though changes in PDH phosphorylation were not significant (Panel B). All values are expressed as mean ± SEM. — indicates significant body size x diet interaction. * Indicates significant response to the diet. # Indicates significant difference from lean. Significance is indicated at $P < 0.05$.

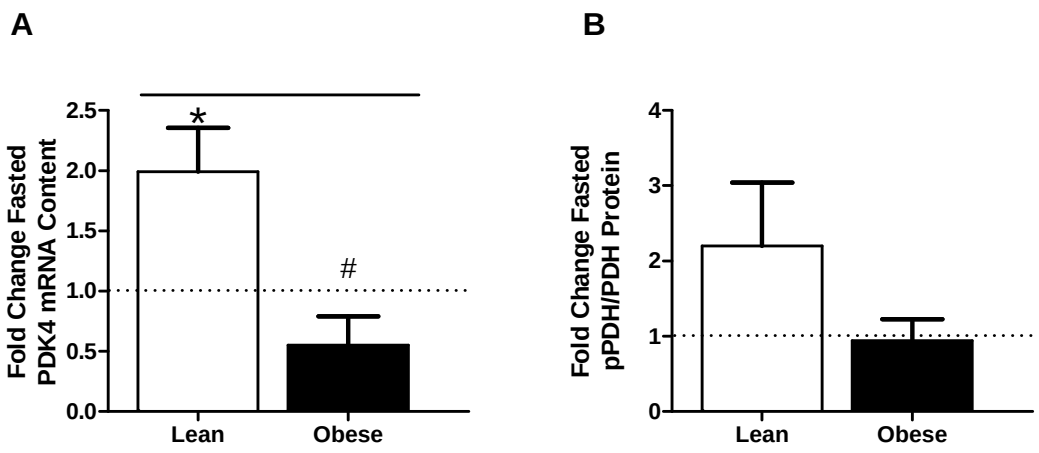

A
B
Fold Change Fasted
PDK4 mRNA Content
2.5
2.0
1.5
1.0
0.5
0.0
*
#
Lean
Obese
Fold Change Fasted
pPDH/PDH Protein
4
3
2
1
0
Lean
Obese

FIGURE 14. BMI AND FASTED INSULIN & CHANGE IN FASTED PDK4 MRNA CONTENT.

Relationships between BMI and change in fasted PDK4 mRNA content (r^2 = 0.273, $P < 0.05$; panel A) and fasted insulin and change in fasted PDK4 mRNA content (r^2 = 0.285, $P < 0.05$; panel B) in lean (o) and obese (•) participants.

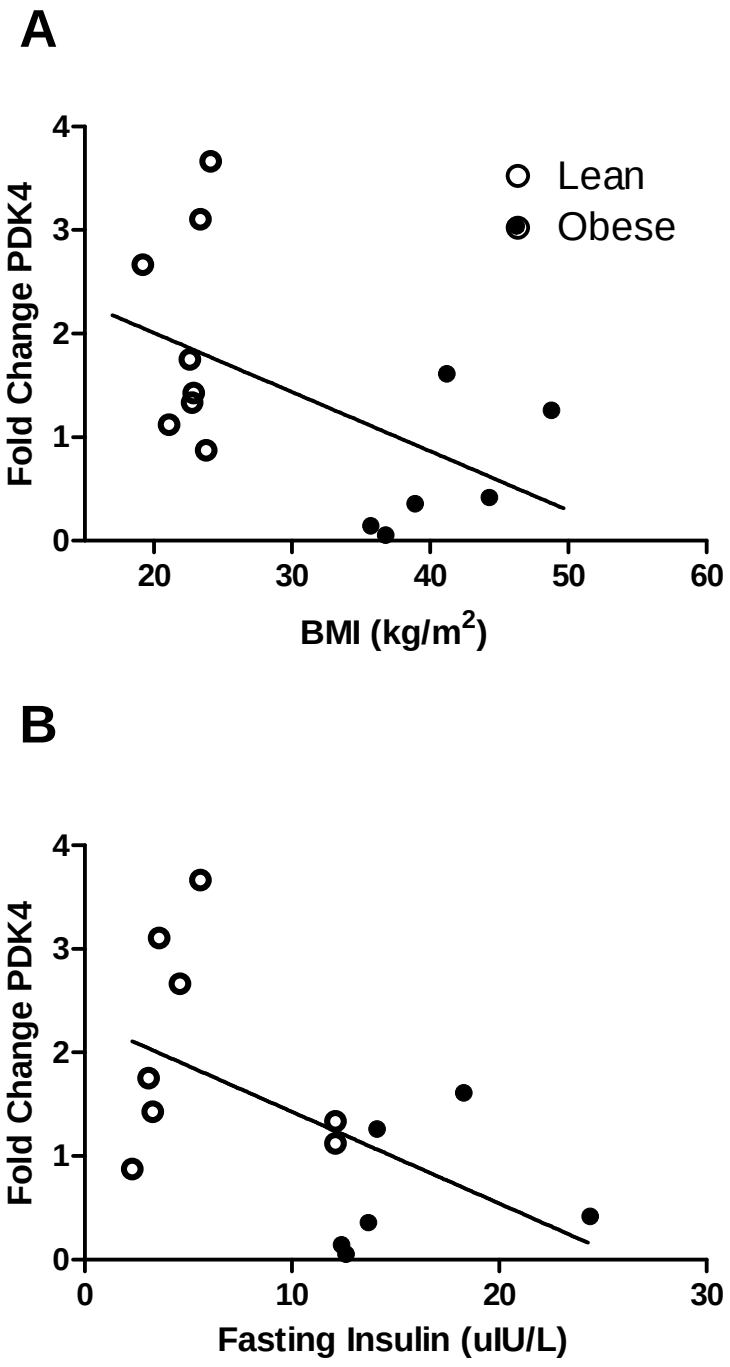
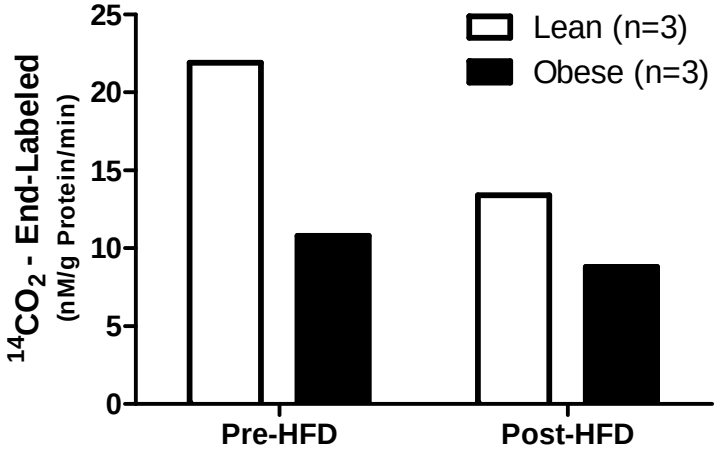

A
Lean
Obese
Fold Change PDK4
BMI (kg/m2)
0
1
2
3
4
20
30
40
50
60
B
Fold Change PDK4
Fasting Insulin (uIU/L)
0
10
20
30

FIGURE 15. SKELETAL MUSCLE PALMITATE OXIDATION PRE- AND POST-HFD.

Pre- and Post-HFD CO_2 production from 1^4C Palmitate, representing total lipid oxidation, infasted tissue samples from lean (open bars) and obese (closed bars) humans.


Lean (n=3)
Obese (n=3)
$^{14}CO_2$ - End-Labeled
(nM/g Protein/min)
25
20
15
10
5
0
Pre-HFD
Post-HFD

APPENDIX C: ADDITIONAL RESULTS – CHAPTER 3

FIGURE 16. CONFOCAL MICROSCOPY IMAGE.

Human skeletal muscle myotubes from one obese donor, stained for mitochondria, visualized using confocoal microscopy.

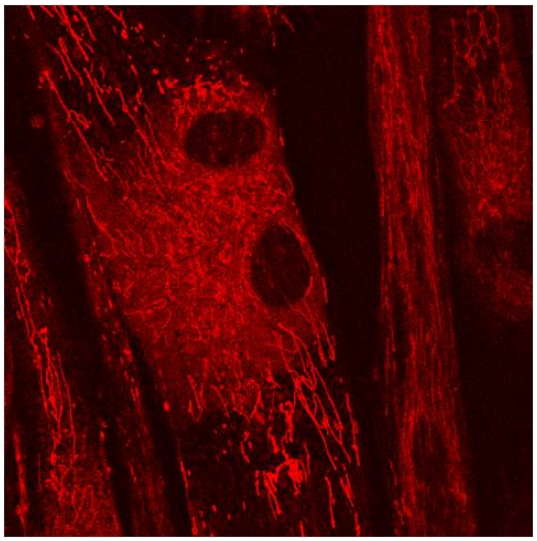
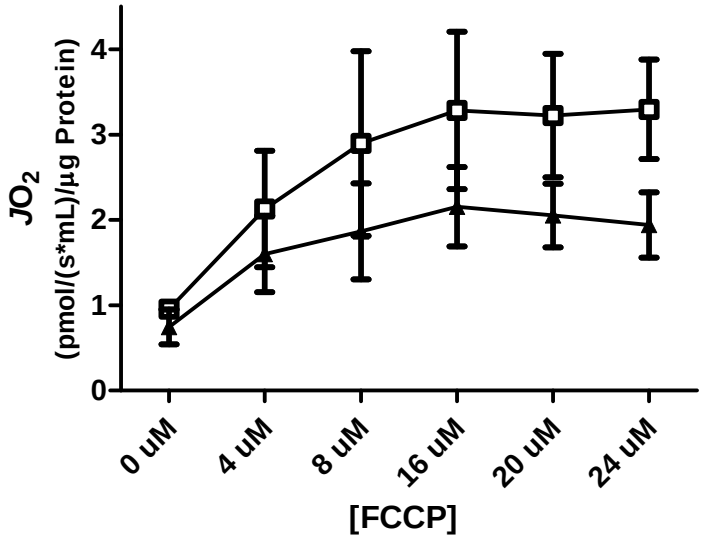


FIGURE 17. BASAL AND UNCOUPLED RESPIRATION IN HSMC FROM LEAN AND OBESE DONORS.

Oxygen flux in whole cell preparations of cultured myotubes from lean (open squares) and obese (closed triangles) human donors. Data are presented during basal conditions (oligomycin) and titrations to maximally uncoupled respiration (FCCP). Data are presented as mean ± SEM.


JO2
(pmol/(s*mL)/µg Protein)
0
1
2
3
4
0 uM
4 uM
8 uM
16 uM
20 uM
24 uM
[FCCP]

APPENDIX D: IRB APPROVAL LETTER

University and Medical Center Institutional Review Board
East Carolina University
Ed Warren Life Sciences Building • 500 Moye Boulevard • LSB 104 • Greenville, NC 27834
Office **252-744-2914** • Fax **252-744-2284** • **www.ecu.edu/irb**
Chair and Director of Biomedical IRB: L. Wiley Nifong, MD
Chair and Director of Behavioral and Social Science IRB: Susan L. McCammon, PhD

TO: Joseph Houmard, PhD, Department of EXSS, ECU, 363 Ward Sports Medicine Bldg.

FROM: UMCIRB

DATE: January 30, 2009

RE: Full Committee Approval for Continuing Review of a Research Study

TITLE: Lipid Metabolism in Obesity, Weigh: Loss and Exercise (2)

UMCIRB #06-0080

The above referenced research study was initially reviewed by the convened University and Medical Center Institutional Review Board (UMCIRB) on **2/8/06**. The research study underwent a subsequent continuing review for approval on **1/28/09** by the convened UMCIRB. The UMCIRB deemed this **NIH** sponsored study **more than minimal risk** requiring a continuing review in **12 months**. 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 above referenced research study has been given approval for the period of **1/28/09** to **1/27/10**. The approval includes the following items:

- Continuing Review Form (dated 1.8.09)
- Informed Consent: Lipid Infusion Study, Version 3
- Informed Consent: High-Fat Diet Study, Version 3
- Informed Consent: Muscle Cell Structure, Version 3
- Informed Consent: Fibrate Drug Study, Version 3
- Informed Consent: Effect of Exercise Training/Lipid Infusion Study, Version 3
- Informed Consent: Effect of Exercise Training/High Fat Diet, Version 3
- Informed Consent: Effect of Weight Loss/Lipid Infusion Study, Version 3
- Informed Consent: Effect of Weight Loss/High Fat Diet, Version 3
- Informed Consent: Effect of Exercise Training, Version 3
- Flyers
- Protocol Summary
- Protocol
- Publications

The following UMCIRB members were recused for reasons of potential for Conflict of Interest on this research study: None

NOTE: The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting: None

The UMCIRB applies 45 CFR 46, Subparts A-D, to all research reviewed by the UMCIRB regardless of the funding source. 21 CFR 50 and 21 CFR 56 are applied to all research studies under the Food and Drug Administration regulation. The UMCIRB follows applicable International Conference on Harmonisation Good Clinical Practice guidelines.