METABOLIC *IN*FLEXIBILITY IN RESPONSE TO LIPID OVERSUPPLY WITH OBESITY: EPIGENETIC MODIFICATIONS PLAY A ROLE

by

Jill Maples

June, 2013

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

Major Department: Department of Kinesiology

The ability to adjust substrate oxidation according to nutrient availability has been termed 'metabolic flexibility' and is a critical factor in overall metabolic health. In respect to fatty acid oxidation (FAO) metabolic flexibility appears to be compromised with severe obesity (BMI > 40kg/m^2). When given a high-fat diet, healthy lean individuals increase their FAO, which is accompanied by increased expression of lipid-oxidizing genes. We observed an impairment in the ability to increase FAO in response to a high-fat diet in the skeletal muscle of obese individuals, which was accompanied by little or no change in the transcriptional upregulation of genes involved in FAO. These data indicate a differential response to lipid oversupply with obesity which could contribute to positive lipid balance and weight gain.

The molecular mechanisms contributing to this metabolic *in*flexibility with severe obesity are not evident. Acute epigenetic modifications of the genome, such as DNA methylation and histone acetylation, may provide a connection between nutritional factors, gene expression, and metabolic health. The purpose of the present study was therefore to determine if the expression of genes linked with FAO differed in a manner indicative of a lack of metabolic flexibility with

obesity and to what extent the differential responses to lipid oversupply were linked with the chromatin environment and/or the methylation signature of these genes.

By utilizing human skeletal muscle cultures (HSkMC) we were able to study the molecular adaptations to a lipid stimulus in the skeletal muscle of lean and obese humans. The main findings were that: 1) the coordinated activation of genes linked with FAO among lean individuals in response to lipid oversupply is largely absent with obesity as evidenced by a blunted upregulation of several vital transcriptional regulators and 2) that changes in CpG methylation, increased histone acetylation, and transcription factor binding accompanied this response, suggesting that acute epigenetic modifications play a role in the lipid-induced upregulation of these genes. These data provide the novel information that with severe obesity the metabolic *in*flexibility evident in response to lipid exposure may be linked with an inability to upregulate transcriptional regulators caused by differential epigenetic modifications.

METABOLIC *IN*FLEXIBILITY IN RESPONSE TO LIPID OVERSUPPLY WITH OBESITY: EPIGENETIC MODIFICATIONS PLAY A ROLE

A Dissertation

Presented to the Faculty of the Department of Kinesiology

East Carolina University

In Partial Fulfillment of the Requirements for the Degree

Doctor of Philosophy

by

Jill Maples

June, 2013

METABOLIC *IN*FLEXIBILITY IN RESPONSE TO LIPID OVERSUPPLY WITH OBESITY:

EPIGENETIC MODIFICATIONS PLAY A ROLE

By

Jill Maples

APPROVED BY:

DIRECTOR OF DISSERTATION/THESIS:_____
(Joseph A. Houmard, Ph.D)

COMMITTEE MEMBER: _____
(Jeffrey J. Brault, PhD)

COMMITTEE MEMBER: _____
(Robert G. Carroll, Ph.D)

COMMITTEE MEMBER: _____
(Brian M. Shewchuk, Ph.D)

COMMITTEE MEMBER: _____
(Carol A. Witczak, Ph.D)

CHAIR OF
THE DEPARTMENT OF KINESIOLOGY:_____
(Stacey R. Altman, Ph.D)

DEAN OF THE COLLEGE OF
HEALTH AND HUMAN PERFORMANCE:_____
(Glen Gilbert, PhD)

DEAN OF
THE GRADUATE SCHOOL:_____
(Paul J. Gemperline, PhD)

DEDICATION

I dedicate this dissertation to my husband, Aaron Maples.

ACKNOWLEDGEMENTS

I would first like to thank my mentor, Dr. Joseph Houmard. He provided me with and helped facilitate amazing opportunities throughout the progression of my graduate career that have enabled me to learn and grow as a researcher. I especially appreciate the training in grantsmanship and writing, which will serve me well in my professional career. Dr. Houmard was very gracious to allow me to pursue some of my own personal scientific interests, for which, I am very thankful.

I would also like to thank my dissertation committee members: Dr. Jeffrey Brault, Dr. Carol Witczak, Dr. Brian Shewchuk, and Dr. Robert Carroll. Dr. Brault was absolutely essential in my scientific training and completion of this project would not have been possible without him. Dr. Witczak shared with me priceless wisdom and advice that helped shape my graduate career. Dr. Shewchuk was kind enough to share his expertise in epigenetics and was always very enthusiastic about the project and helping me when needed. Dr. Carroll helped me see how grants and manuscripts that have a logical flow are constructed. Together, my committee made this experience the absolute best that I could have imagined.

Thank you to Dr. Monica Hubal for facilitating the epigenetic and comprehensive mRNA analyses, processing the data, and helping to interpret the findings. I am so appreciative of the hours you spent working on the data and patiently teaching me how to work with the data.

I want to thank all of the HPL and Bioenergetics faculty and staff for helping me during my time here at ECU. In particular, Wendy Beachum for all your hard work that was certainly not limited to, but included facilitating the ordering process and working out administrative details. Patty Brophy, Gabe Dubis, Dr. Donghai Zheng, Chuck Tanner, Dr. Robert Hickner, and

Dr. Darrell Neufer all helped play an important role in the development of this dissertation and my graduate education.

Thank you to my close friend Laura Gilliam for being personally supportive and encouraging, as well as essential in my scientific development. I must also acknowledge my wonderful Bioenergetics colleagues and fellow students: Gina Battaglia, Kelsey Fisher-Wellman, Dan Lark, Maria Torres, Jeremie Ferey, Sanghee Park, Patrick Davis, Lance Bollinger, Matt Hinkley, and Cody Smith. I especially would like to thank Kathleen Gavin, Todd Weber, and Joe Pierce for their scholarly advice, making work more enjoyable, and for their friendship.

Finally, I would like to thank my wonderful and supportive family. My husband, Aaron Maples, is the most amazing man that I have ever met and I am so thankful for the extraordinary marriage that we share. My mother, Janat VanMeter, and sister, Emily Blevens, for their love and support. The entire Maples Family, including Ron, Susan, Julie, Marly, and Jim, I am so fortunate to be a part of this family.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

ANGPTL4: angiopoieten-like 4

ANOVA: analysis of variance

BMI: body mass index

BSA: bovine serum albumin

COX6c: cytochrome c oxidase subunit VIc

CPT1: carnitine palmitoyl transferase-1

CPT1A: carnitine palmitoyl transferase-1 isoform A

CPT1B: carnitine palmitoyl transferase-1 isoform B

CPT1C: carnitine palmitoyl transferase-1 isoform C

CS: citrate synthase

CYCS: cytochrome c

DMEM: Dulbecco Modified Eagle's Medium

DNA: deoxyribonucleic acid

FAO: fatty acid oxidation

GFM1: mitochondrial elongation factor G 1

HFD: high-fat diet

HNF-4α: hepatocyte nuclear factor 4

HOMA-IR: homeostasis model of assessment-insulin resistance

HSkMC: primary human skeletal muscle cells raised in culture

mRNA: messenger ribonucleic acid

MRPL2: mitochondrial ribosomal protein L 2

NRF: nuclear respiratory factor

NRF-1: nuclear respiratory factor 1

NRF-2: nuclear respiratory factor 2

PCR: polymerase chain reaction

PDK4: pyruvate dehydrogenase kinase 4

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

PGC-1β: peroxisome proliferator-activated receptor gamma coactivator beta

PPAR: peroxisome proliferator-activated receptor

PPARα: peroxisome proliferator-activated receptor alpha

PPARβ: peroxisome proliferator-activated receptor beta

PPARδ: peroxisome proliferator-activated receptor delta

PPRC1: peroxisome proliferator-activated receptor gamma coactivator-related protein 1

RNA: ribonucleic acid

SEM: standard error of the mean

TFAM: mitochondrial transcription factor A

TFB2M: mitochondrial transcription factor B 2

UCP3: mitochondrial uncoupling protein 3

CHAPTER 1: LITERATURE REVIEW

PREVALENCE AND IMPACT OF SEVERE OBESITY

In recent decades, the steady increase in the prevalence of obesity across all sex and age groups was significant (1). Some reports indicate that the increase in prevalence of obesity over the last decade has slowed, however as of 2008 32.2% of US adults are class I obese (BMI $\geq$ 30 kg/m^2), 14.3% are class II obese (BMI $\geq$ 35 kg/m^2), and 5.6% are class III obese (BMI $\geq$ 40 kg/m^2), which is also referred to as "severely" or "extremely" obese (1, 2). When the prevalence trends for the higher classes of obesity are examined, it is clear that the prevalence of extreme obesity is increasing at alarming rates. According to national Behavioral Risk Factor Survey (BRFS) results, from 2000 to 2005 the prevalence of BMI $\geq$ 40 kg/m^2 increased by 52%, while the prevalence of BMI $\geq$ 50 kg/m^2 increased 75% (3).

The increase in prevalence of extreme cases of obesity is associated with increased health care economic burden. This economic burden is disproportionately large for the most obese. For example, compared to an individual with a healthy weight status (BMI 18.5-24.9 kg/m^2), individuals with a BMI between 35-40 kg/m^2 spend 50% more, while individuals with a BMI greater than 40 kg/m^2 doubled health care spending relative to an individual with a healthy weight status (4). Increased health care spending is a combination of more expensive office and emergency department visits as well as prescription medication costs. For example, medical treatment of extremely obese individuals often requires specialized equipment, supplies, and additional support staff (5).

In addition to increased economic burden, it is well known that severe obesity is associated with an increased mortality rate and a variety of chronic diseases including

cardiovascular and metabolic diseases (i.e. insulin resistance, type 2 diabetes, etc.) (2). There is accumulating evidence that these metabolic diseases are associated with a defect in the ability of skeletal muscle to oxidize lipids (6) and an inability to adjust substrate oxidation according to nutrient availability, which has been termed 'metabolic flexibility' and is a critical factor in overall metabolic health (7).

METABOLIC *IN*FLEXIBILITY WITH SEVERE OBESITY

In respect to fatty acid oxidation (FAO) metabolic flexibility appears to be compromised with obesity. For example, several studies have reported an impairment in the ability to increase FAO in response to a 3-5d high-fat diet in the skeletal muscle of obese individuals (8, 9); this phenotype was also apparent with lipid incubation in primary human skeletal muscle cells raised in culture (HSkMC) (10). When given a high-fat diet, healthy lean individuals increase their FAO (9, 11), which is accompanied by increased expression of mitochondrial and lipid-oxidizing genes (e.g. peroxisome proliferator-activated receptor (*PPAR*)α and PPARγ coactivator-1α (*PGC-1α*)) (8, 10-14), while a high fat diet resulted in little to no change in the expression of these genes in obese subjects (8). These data indicate a differential response to lipid oversupply with obesity which likely contributes to positive lipid balance, ectopic lipid accumulation, intramuscular lipid accumulation associated with insulin resistance, weight gain, and weight regain after weight loss (15, 16).

The molecular mechanisms contributing to this metabolic *in*flexibility with severe obesity, however, are not evident. Epigenetic modifications provide a potential molecular basis for the interaction between genetic and environmental factors and have been implicated in metabolic diseases, stroke, and cardiovascular disease (17-20). Acute epigenetic modifications of the

genome, such as DNA methylation and histone acetylation, may provide a connection between nutritional factors, gene expression, and metabolic health and could help explain the differential responses in the coordinated gene expression in the skeletal muscle of lean vs. severely obese individuals. DNA methylation is generally accepted to regulate gene transcription by directly impeding the binding of transcriptional factors to their target sites and through the recruitment of methyl-binding proteins (21). Histone acetylation affects both chromatin structure as well as the interaction of transcription-regulatory proteins with target DNA in chromatin (22). For example, an increase in the acetyl groups on histones will result in an open chromatin structure to facilitate accessibility of transcriptional machinery to DNA templates in chromatin, in turn increasing the expression of a gene. There is a large body of evidence on the functional significance of both histone acetylation and DNA methylation levels and their correlation with gene expression as well as their importance in integrating environmental stimuli, such as diet, in the control of gene expression (21, 22). For example, with a 5 d high fat diet Jacobsen et al. (23) observed widespread DNA methylation changes in human skeletal muscle and Barres et al. (17) reported increased non-CpG methylation of the *PGC-1α* promoter region in HSkMC with a 48hr 500µM palmitate incubation. However, these studies (9,10,11) examined skeletal muscle from lean individuals; it is not evident if differences in DNA methylation patterns may explain, at least in part, the divergent responses to lipid of genes involved with mitochondrial biogenesis and FAO in the skeletal muscle of obese vs. lean individuals (3).

ARE EPIGENETIC MODIFICATIONS CONTRIBUTING TO METABOLIC *IN*FLEXIBILITY?

This dissertation focused on determining whether the differential responses to lipid oversupply of genes linked with mitochondrial content and FAO with severe obesity are linked with epigenetic modifications, including DNA methylation and histone acetylation, in a manner indicative of a lack of metabolic flexibility. The nuclear respiratory factors (NRFs) and peroxisome proliferator-activated receptors (PPARs) and are provocative candidates for explaining the metabolic inflexibility in response to lipid oversupply with obesity as they activate gene expression programs critical to mitochondrial function and FAO (24-27).

The NRFs are required for the expression of the respiratory apparatus in mammalian cells and vital for mitochondrial biogenesis and maintenance as indicated by early mortality of *NRF-1*-null embryos (24). The PPARs are ligand-activated transcription factors that play essential roles in lipid homeostasis by modulating the expression of genes that regulate fatty acid catabolism. One particularly attractive PPAR-regulated candidate is carnitine palmitoyltransferase (CPT1) which mediates the transfer of long chain fatty acids across the outer mitochondrial membrane which is a rate-limiting step in FAO (28). The activity of the muscle-type CPT1 (encoded by the *CPT1B* gene) is reduced in skeletal muscle with obesity, contributing to a decrease in FAO (29). In relation to metabolic flexibility, in the skeletal muscle of lean subjects a high fat diet increased the expression of *CTP1B* and the peroxisome proliferator-activated receptors (*PPAR*s) which are upstream regulators of *CPT1B* expression (8). Conversely, a high fat diet resulted in little to no change in the expression of these genes in the

skeletal muscle of severely obese subjects indicating a differential response to lipid oversupply with severe obesity (8).

Therefore, the specific purpose of this dissertation was to determine if the expression of PPAR- and/or NRF-regulated genes linked with FAO and mitochondrial content differed in a manner indicative of a lack of metabolic flexibility with severe obesity and to what extent the differential responses to lipid oversupply were linked with the chromatin environment and/or the methylation signature of these genes. By utilizing HSkMC we were able to study the molecular adaptations to a lipid stimulus in an environment void of *in-vivo* hormonal and neural stimuli and thus intrinsic to skeletal muscle itself.

REFERENCES

1. Flegal KM, Carroll MD, Ogden CL, Curtin LR**.** Prevalence and trends in obesity among US adults, 1999-2008. Jama. 2010;303(3):235-41. Epub 2010 Jan 13.

2. NHLBI**.** Executive summary of the clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults. Arch Intern Med. 1998;158(17):1855-67.

3. Sturm R**.** Increases in morbid obesity in the USA: 2000-2005. Public Health. 2007;121(7):492-6. Epub 2007 Mar 30.

4. Andreyeva T, Sturm R, Ringel JS**.** Moderate and severe obesity have large differences in health care costs. Obes Res. 2004;12(12):1936-43.

5. Geiling J**.** Critical care of the morbidly obese in disaster. Crit Care Clin. 2010;26(4):703-14. Epub 2010 Aug 8.

6. Consitt LA, Bell JA, Houmard JA**.** Intramuscular lipid metabolism, insulin action, and obesity. IUBMB Life. 2009;61(1):47-55.

7. Kelley DE, Mandarino LJ**.** Fuel selection in human skeletal muscle in insulin resistance: a reexamination. Diabetes. 2000;49(5):677-83.

8. Boyle KE, Canham JP, Consitt LA, Zheng D, Koves TR, Gavin TP, et al. A high-fat diet elicits differential responses in genes coordinating oxidative metabolism in skeletal muscle of lean and obese individuals. J Clin Endocrinol Metab. 2011;96(3):775-81. Epub 2010 Dec 29.

9. Battaglia GM, Zheng D, Hickner RC, Houmard JA**.** Effect of exercise training on metabolic flexibility in response to a high-fat diet in obese individuals. Am J Physiol Endocrinol Metab. 2012;303(12):E1440-5. doi: 10.152/ajpendo.00355.2012. Epub 2012 Oct 9.

10. Boyle KE, Zheng D, Anderson EJ, Neufer PD, Houmard JA**.** Mitochondrial lipid oxidation is impaired in cultured myotubes from obese humans. Int J Obes. 2011;25(10):201.

11. Cameron-Smith D, Burke LM, Angus DJ, Tunstall RJ, Cox GR, Bonen A, et al. A short-term, high-fat diet up-regulates lipid metabolism and gene expression in human skeletal muscle. Am J Clin Nutr. 2003;77(2):313-8.

12. Crunkhorn S, Dearie F, Mantzoros C, Gami H, da Silva WS, Espinoza D, et al. Peroxisome proliferator activator receptor gamma coactivator-1 expression is reduced in obesity: potential pathogenic role of saturated fatty acids and p38 mitogen-activated protein kinase activation. J Biol Chem. 2007;282(21):15439-50. Epub 2007 Apr 6.

13. Hancock CR, Han DH, Chen M, Terada S, Yasuda T, Wright DC, et al. High-fat diets cause insulin resistance despite an increase in muscle mitochondria. Proc Natl Acad Sci U S A. 2008;105(22):7815-20. Epub 2008 May 28.

14. Hock MB, Kralli A. Transcriptional control of mitochondrial biogenesis and function. Annu Rev Physiol. 2009;71:177-203.

15. Consitt LA, Bell JA, Koves TR, Muoio DM, Hulver MW, Haynie KR, et al. Peroxisome proliferator-activated receptor-gamma coactivator-1alpha overexpression increases lipid oxidation in myocytes from extremely obese individuals. Diabetes. 2010;59(6):1407-15. Epub 2010 Mar 3.

16. Zurlo F, Lillioja S, Esposito-Del Puente A, Nyomba BL, Raz I, Saad MF, et al. Low ratio of fat to carbohydrate oxidation as predictor of weight gain: study of 24-h RQ. Am J Physiol. 1990;259(5 Pt 1):E650-7.

17. Barres R, Osler ME, Yan J, Rune A, Fritz T, Caidahl K, et al. Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density. Cell Metab. 2009;10(3):189-98.

18. Lund G, Zaina S. Atherosclerosis: an epigenetic balancing act that goes wrong. Curr Atheroscler Rep. 2011;13(3):208-14.

19. Qureshi IA, Mehler MF. Emerging role of epigenetics in stroke: part 1: DNA methylation and chromatin modifications. Arch Neurol. 2010;67(11):1316-22.

20. Shirodkar AV, Marsden PA. Epigenetics in cardiovascular disease. Curr Opin Cardiol. 2011;26(3):209-15.

21. Miranda TB, Jones PA. DNA methylation: the nuts and bolts of repression. J Cell Physiol. 2007;213(2):384-90.

22. Vaissiere T, Sawan C, Herceg Z. Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat Res. 2008;659(1-2):40-8. Epub 2008 Feb 29.

23. Jacobsen SC, Brons C, Bork-Jensen J, Ribel-Madsen R, Yang B, Lara E, et al. Effects of short-term high-fat overfeeding on genome-wide DNA methylation in the skeletal muscle of healthy young men. Diabetologia. 2012;55(12):3341-9. doi: 10.1007/s00125-012-2717-8. Epub 2012 Sep 8.

24. Kelly DP, Scarpulla RC**.** Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev. 2004;18(4):357-68.

25. Muoio DM, MacLean PS, Lang DB, Li S, Houmard JA, Way JM, et al. Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) alpha knock-out mice. Evidence for compensatory regulation by PPAR delta. J Biol Chem. 2002;277(29):26089-97. Epub 2002 May 6.

26. Lee CH, Olson P, Evans RM**.** Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. Endocrinology. 2003;144(6):2201-7.

27. Muoio DM, Koves TR**.** Skeletal muscle adaptation to fatty acid depends on coordinated actions of the PPARs and PGC1 alpha: implications for metabolic disease. Appl Physiol Nutr Metab. 2007;32(5):874-83.

28. McGarry JD, Brown NF**.** The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem. 1997;244(1):1-14.

29. Kim JY, Hickner RC, Cortright RL, Dohm GL, Houmard JA**.** Lipid oxidation is reduced in obese human skeletal muscle. Am J Physiol Endocrinol Metab. 2000;279(5):E1039-44.

CHAPTER 2: SKELETAL MUSCLE FROM THE SEVERELY OBESE IS METABOLICALLY *IN*FLEXIBLE IN TERMS OF GENE REGULATION AND DNA METHYLATION IN RESPONSE TO LIPID OVERSUPPLY

ABSTRACT

Objective: The skeletal muscle of severely obese individuals (BMI $\geq$ 40 kg/m^2) is characterized by a failure to upregulate fatty acid oxidation (FAO) in response to increased lipid availability, which is associated with a failure to coordinately upregulate genes involved in FAO. The present study was undertaken to determine if the differential responses to lipid oversupply of genes linked with mitochondrial content and FAO with severe obesity are linked with the methylation signatures of these genes.

Research design and methods: RNA and DNA were isolated in human skeletal muscle cell cultures from 9 lean and 10 severely obese women following lipid incubation. mRNA content was measured using RT-PCR. Methylation was quantified using the Illumina HumanMethylation450 BeadChip.

Results: The upregulation of several vital transcriptional regulators of FAO and mitochondrial content were depressed in the severely obese compared to their lean counterparts in response to lipid oversupply and there is evidence that the expression of *PPARδ*, which regulates a broad transcriptional program related to energy metabolism, is controlled by changes in CpG methylation.

Conclusions: With severe obesity, the metabolic *in*flexibility evident with lipid exposure is linked with an inability to upregulate several transcriptional regulators and methylation may play a role in this transcriptional regulation.

INTRODUCTION

The ability to adjust substrate oxidation according to nutrient availability has been termed 'metabolic flexibility' and is a critical factor in overall metabolic health (7). In respect to fatty acid oxidation (FAO), metabolic flexibility appears to be compromised with obesity. For example, several studies have reported an impairment in the ability to increase FAO in response to a 3-5d high-fat diet in the skeletal muscle of obese individuals (8, 9); this phenotype was also apparent with lipid incubation in primary human skeletal muscle cells raised in culture (HSkMC) (10). Additionally, in the skeletal muscle of lean subjects a high-fat diet increased the expression of genes involved in mitochondrial biogenesis and FAO (i.e. peroxisome proliferator-activated receptor (*PPAR*)α and PPARγ coactivator-1α (*PGC-1α*)) (8, 10-14), while a high-fat diet resulted in little to no change in the expression of these genes in obese subjects (8). These data indicate a differential response to lipid oversupply with obesity which could contribute to positive lipid balance and ectopic lipid accumulation (15, 16, 30, 31).

Acute epigenetic modifications of the genome, such as DNA methylation, may provide a connection between nutritional factors, gene expression, and metabolic health. For example, with a 5d high-fat diet Jacobsen et al. (23) observed widespread DNA methylation changes in human skeletal muscle and Barres et al. (17) reported increased non-CpG methylation of the *PGC-1α* promoter region in HSkMC with a 48hr 500μM palmitate incubation. However, these studies (17, 23) examined skeletal muscle from lean individuals; it is not evident if differences in DNA methylation patterns may explain, at least in part, the divergent responses to lipid of genes involved with mitochondrial biogenesis and FAO in the skeletal muscle of obese vs. lean individuals. The purpose of the present study was therefore to determine if the differential

responses to lipid oversupply of genes linked with mitochondrial content and FAO with obesity are linked with the methylation signatures of these genes. By utilizing HSkMC we are able to study the molecular adaptations to a lipid stimulus in an environment void of *in-vivo* hormonal and neural stimuli and thus intrinsic to skeletal muscle itself.

RESEARCH DESIGN AND METHODS

Study Design

The design of the study was to compare the responses of genes linked with FAO and mitochondrial biogenesis to lipid oversupply in lean vs. obese subjects. Skeletal muscle was obtained from the vastus lateralis and used to derive primary human skeletal muscle cell cultures (HSkMC). After differentiation into myotubes, HSkMC were incubated in a physiologically relevant lipid mixture (32) (250μM oleate:palmitate) for 48hr and mRNA content and DNA methylation determined. The mRNA content of the peroxisome proliferator-activated receptors (PPARs) and nuclear respiratory factors (NRFs) were selected to be analyzed because of their importance in activating gene expression programs critical to mitochondrial function and FAO (24-27). Additionally, downstream PPAR and NRF-regulated genes that play vital roles in mitochondrial content and FAO were analyzed as well.

Materials

All chemical reagents and substrates were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Dulbecco's Phosphate-Buffered Saline (DPBS), fetal bovine serum, heat-inactivated horse serum, gentamicin, 0.05% trypsin EDTA, and Hanks's balanced salt solution were obtained from Invitrogen. Growth media and differentiation media consisted of low glucose (5 mmol/L) Dulbecco's Modified Eagles Medium from Invitrogen. Type I collagen-coated tissue culture plates were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). PCR reagents were purchased from Applied Biosystems (Foster City, CA, USA).

Human Subjects

Muscle biopsies were obtained using the percutaneous needle biopsy technique (33) under local anesthesia (0.01% lidocaine) from the vastus lateralis of 9 lean (BMI= 22.8 kg/m^2 ±2.2; Age= 23.4yrs ±4.6) and 10 obese (Class II to III) (BMI= 41.3 kg/m^2 ±4.9; Age= 30.2yrs±8.3) Caucasian women. Characteristics are presented in Table 1. Participants were relatively young, free from overt disease, nonsmokers and not taking medications known to alter metabolism. All procedures were approved by the East Carolina University Institutional Review Board.

Primary HSkMC

Satellite cells were isolated from ~50-100mg of fresh muscle tissue and cultured as previously described (34). For experiments cells were sub-cultured into T-150 flasks and 10cm dishes. Upon reaching ~80-90% confluence, differentiation was induced by switching the growth media to low-serum differentiation media containing 2% heat-inactivated horse serum, 0.05 mg/ml fetuin, and 5 µg/ml gentamicin. On day 5 of differentiation, myotubes were given fresh differentiation media supplemented with 1) 0.1% bovine serum albumin (BSA) + 1mM carnitine (CONTROL) or 2) 250µM oleate:palmitate (1:1 ratio) bound to 0.1% BSA + 1mM carnitine (LIPID) for a total incubation period of 48 hours. Myotubes were harvested on day seven similar to previous work (35). There were no obvious differences in the extent of differentiation or myotube morphology between lean and obese HSkMC.

RNA Isolation and mRNA quantification

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) with on-column DNase digestion using the RNase-Free DNase Set (Qiagen, Valencia, CA) to remove residual DNA. RNA was quantified using the NanoDrop 1000 Spectrophotometer Version 3.7.1 from Thermoscientific (Wilmington, DE, USA) and concentration was determined by measuring the absorbance at 260nm. 2ug of RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA, USA). PCR was performed in triplicate using the Applied Biosystems ABI 7900HT sequence detection instrument and software with Taqman Universal PCR Master Mix and TaqMan gene expression assays (Applied Biosystems, Foster City, California) in accordance with manufacturer's instructions. Using standard techniques, reactions were run with the following thermal cycling conditions: 50˚C for 2 min; 95˚C for 10 min; and 40 cycles of 95˚C for 15 s; followed by 60˚C for 1 min. mRNA content was measured using the comparative Ct method with a multiplexed endogenous control (18S) and converted to a linear function by using a base 2 antilog transformation.

DNA Isolation

Cells were washed with DPBS and trypsinized with trypsin-EDTA (0.05% trypsin and 0.25% EDTA). Total DNA (mitochondrial and nuclear) was extracted from cells using a QIAamp DNA mini kit (Qiagen, Valencia, CA), and total DNA quantified using the NanoDrop 1000 Spectrophotometer Version 3.7.1 from Thermoscientific (Wilmington, DE, USA).

Bisulfite Conversion and DNA Methylation Profiling

500ng of DNA was bisulfate converted using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) following the manufacturer's protocol, with the alternative incubation conditions recommended when using the Illumina Infunium Methylation Assay. Genome-wide DNA methylation analysis was conducted on bisulfate treated DNA samples using the Illumina Infinium HumanMethylation 450K BeadChip, which allows the quantitative monitoring of 485,764 cytosine positions (36). 12uL of each bisulfate-converted sample was amplified and fragmented, following the manufacturer's protocol, hybridized to arrays in a balanced design and scanned on an Illumina iScan System. Data were output and analyzed using Illumina's Genome Studio software.

Statistical analysis

Statistical analyses were performed using PASW Statistics 19 Software (SPSS Inc., Chicago, IL, USA) on raw or log-transformed data. Comparisons between HSkMC from lean and obese donors were performed with repeated measures ANOVA, with emphasis on a "weight status" (lean, severely obese) X "treatment" (control, lipid-treated) interaction indicating that lean and severely obese individuals responded differently to lipid oversupply. All data met assumptions of sphericity and homogeneity of variance. Data are presented as the mean ± SEM.

RESULTS

Participant Characteristics

Participant characteristics are presented in Table 1. By design, the obese subjects were heavier and most had a body mass index (BMI) classified as Class III (severe) obesity (≥40 kg/m^2). Fasting blood glucose, cholesterol, and triglyceride values did not differ between groups. However fasting HDL values were significantly lower, while insulin and homeostatic model assessment values were significantly higher in the obese subjects. Both groups consisted of relatively young individuals, however, the age of the obese group was higher compared to the lean group (P=0.05).

Gene Expression

Under the control condition (i.e. pre-lipid exposure), there were no differences between the groups in *PPARα*, *NRF-1*, and *NRF-2* mRNA content; however, *PPARδ* mRNA content was significantly lower (P=0.04) in the obese individuals compared to the lean (Fig. 2.2). In response to the 48h 1:1 oleate:palmitate lipid treatment, there were significant ($P \leq 0.05$) interaction effects for *PPARα*, *PPARδ*, *NRF-1*, and *NRF-2* where mRNA content was elevated in the lipid-treated state in HSkMC from the lean, but not obese subjects (Fig. 2.1 and 2.2). There were no differences in the mRNA content between groups in either condition for *PGC-1α, PGC-1β*, and peroxisome proliferator-activated receptor gamma coactivator-related protein 1 (*PPRC1*) (Fig. 2.1).

In an effort to determine whether the differential expression of these transcriptional regulators had a downstream effect, four PPAR-responsive genes that play a vital role in FAO

were analyzed (Table 2.2) angiopoieten-like 4 (*ANGPTL4*), 2) citrate synthase (*CS*), 3) pyruvate dehydrogenase kinase 4 (*PDK4*), and 4) mitochondrial uncoupling protein 3 (*UCP3*). There was a significant interaction effect for *PDK4* ($P \leq 0.05$) with the lipid-induced change in mRNA being significantly lower ($P \leq 0.05$) in the HSkMC from the obese (20.4 ±3.8 fold increase) compared to the lean (42.0±8.1 fold increase) subjects (Fig. 2.3A). Similarly, the lipid-induced increase in *CS* and *UCP3* mRNA content in HSkMC from the obese (1.2 ±0.2 and 1.2±0.1 fold increase) compared to the lean (1.6±0.1 and 0.9±0.1 fold increase) exhibited a trend ($P=0.1$ and $P=0.09$) for being suppressed with obesity. Six NRF regulated genes were analyzed (Table 2.2): 1) cytochrome c oxidase subunit VIc (*COX6c*), 2) cytochrome c (*CYCS*), 3) mitochondrial elongation factor G 1 (*GFM1*), 4) mitochondrial ribosomal protein L2 (*MRPL2*), 5) mitochondrial transcription factor A (*TFAM*), and 6) mitochondrial transcription factor B2 (*TFB2M*). The lipid-induced increase in *CYCS* mRNA content was significantly ($P=0.05$) lower in the obese (0.9±0.1 fold increase) compared to the lean (1.3±0.2 fold increase) subjects (Fig. 2.3B).

DNA Methylation

Overall, DNA methylation was determined on 485,764 cytosine positions. In terms of genes exhibiting differential responses with obesity (Fig. 2.2) within the *PPARδ* gene DNA methylation was determined in 23 sites (Fig. 2.4A), with nine cytosines being within 1000 base pairs (bp) of the transcription start site (TSS). In response to the lipid oversupply, there were significant ($P \leq 0.05$) interaction effects for cytosines at positions 6 and 7 (-71 and -61bp relative to the TSS) in *PPARδ* where methylation increased in the lipid-treated state in HSkMC from the lean, but not obese subjects. From baseline, lipid oversupply resulted in a significant ($P \leq 0.05$)

increase in the methylation of three cytosines (at positions 6, 7, and 23) in HSkMC from lean but not obese women (Fig. 2.4B and C). Lipid oversupply resulted in a similar, significant ($P \leq 0.05$) increase in the methylation of the cytosine at position 14 (41,044bp relative to the TSS) in both the lean and obese subjects (Fig. 2.4B and C). When the absolute methylation percentage of the *PPARδ* gene with lipid treatment was compared between the lean and obese subjects, lipid oversupply resulted in a significantly lower ($P \leq 0.05$) percentage of methylation among two cytosines at positions 6 and 7 in HSkMC from the obese compared to the lean (Fig. 2.5). Additionally, there was a trend for the methylation of two additional cytosines at positions 4 ($P=0.08$) and 5 ($P=0.06$) (-78 and -75bp relative to the TSS) to be lower in the lean compared to the obese in the lipid-treated condition. With lipid treatment methylation of the cytosine at position 6 (-71bp from TSS) significantly increased by an average of 1.5±0.6% in the lean and decreased an average of 0.6±0.3% in the obese, which was positively correlated ($r=0.64$, $P=0.01$) with the relative change in *PPARδ* mRNA content in response to lipid treatment (Fig. 2.6A). With lipid treatment the methylation of the cytosine at position 6 was 6.4±0.7% in the lean and 4.3±0.3% in the obese (Table S2.1), which positively correlated ($r=0.53$, $P=0.03$) with *PPARδ* mRNA content measured in the lipid-treated condition (Fig. 2.6B). Methylation did not change significantly in any of the other differentially expressed genes examined in this study.

DISCUSSION

With obesity, there are impairments in the ability to adjust substrate utilization to changes in substrate availability (37-39). In respect to lipid metabolism, we reported that obese individuals lacked the ability to increase fat oxidation with either a high fat diet (9) or in HSkMC upon lipid incubation (10). In the present study, we examined if the expression of genes linked with FAO and mitochondrial content also differed in a manner indicative of a lack of metabolic flexibility with obesity and if these differences in gene expression could be explained by methylation signatures. The main findings were that: 1) the upregulation of several vital transcriptional regulators of FAO and mitochondrial content were depressed in the severely obese women compared to their lean counterparts in response to lipid oversupply and 2) that the expression of *PPARδ*, which regulates a broad transcriptional program related to energy metabolism, may be controlled by changes in CpG methylation. These data provide the novel information that with severe obesity the metabolic *in*flexibility evident with lipid exposure may be linked with an inability to upregulate some transcriptional regulators via methylation.

The peroxisome proliferator-activated receptors (PPARs) and nuclear respiratory factors (NRFs) are provocative candidates for explaining the metabolic *in*flexibility in response to lipid oversupply with obesity as they activate gene expression programs critical to mitochondrial function and FAO (24-27). The PPARs are ligand-activated transcription factors that play essential roles in lipid homeostasis by modulating the expression of genes that regulate fatty acid catabolism. There are three PPAR subtypes: 1) PPARα, which mediates lipid-induced activation of FAO genes and is expressed predominately in tissues that are characterized by high rates of FAO (ie. liver, heart, muscle, kidney); 2) PPARγ, which is highly enriched in adipocytes and

macrophages and is involved in adipocyte differentiation, lipid storage, and glucose homeostasis; and 3) PPARδ, which is ubiquitously expressed and has the least defined function, but has recently been characterized as being highly expressed in skeletal muscle and playing a lipid-metabolizing role similar to PPARα (25, 26, 40). Their critical role in energy homeostasis is supported by the observation that *PPARα* knockout mice exhibit a dramatic inhibition of fatty acid uptake and oxidation, abnormal accumulation of lipids in oxidative tissues, and a failure to induce beta-oxidation in response to physiological challenges such as a high-fat diet (35). The NRFs are required for the expression of the respiratory apparatus in mammalian cells and vital for mitochondrial biogenesis and maintenance, as indicated by early mortality of *NRF-1*-null embryos (24). In the current study, broad transcriptional regulators, including *PPARα, PPARδ, NRF-1,* and *NRF-2*, exhibited similar patterns of increasing mRNA content with lipid exposure in the lean, but decreasing or not changing mRNA in HSkMC from the severely obese (Fig. 1). Additionally, several NRF- and PPAR-responsive genes including *CYCS, CS, UCP3*, and *PDK4* showed trends for being upregulated more robustly in HSkMC from lean but not obese subjects. We cannot discount the possibility that these findings may have been influenced by the time we chose to obtain the samples, i.e. that similar mRNA responses in lean and obese subjects could have occurred at earlier or later time points during lipid incubation. However, the present data remain indicative of a coordinated lipid-induced activation of genes linked with FAO and mitochondrial content among lean individuals in response to lipid oversupply that is largely absent with obesity

Of the PPAR-responsive genes that were differentially regulated by the 48hr lipid oversupply pyruvate dehydrogenase kinase 4 (*PDK4*) is of particular interest as it suppresses glucose and promotes fat oxidation in the presence of lipids (41). We have previously shown

that a high-fat diet increased *PDK4* mRNA content in lean, but not obese individuals; in the current study utilizing HSkMC, the lipid-induced increase in *PDK4* mRNA content in the lean was more than 2-fold greater than the response in the obese (42.0±8.1 vs. 20.4±3.8 fold increase) (Fig. 3). Other data have reported a significant interaction effect in response to a high fat diet where the increase in *PDK4* mRNA content was more pronounced in obese individuals relative to lean (42). A possible reason for these divergent findings is that the current study utilized HSkMC from severely obese women (Class II to III) (BMI= 41.3 kg/m^2 ±4.9), while the analysis in Bergouignan et al. (42) measured skeletal muscle mRNA content isolated from men and women with a lower mean BMI (35.1 kg/m^2 ±4.1). This suggests that the ability to adjust to substrate availability may depend on the level of excess adiposity (42), which is in agreement with other data indicating that disturbances in lipid metabolism are evident in the skeletal muscle of extremely obese, but not moderately obese individuals (43). PDK4 protein content is primarily regulated via transcription (44); thus although PDK4 content was not determined, such a lack of metabolic flexibility could contribute to detrimental conditions such as positive fat balance (30), ectopic lipid accumulation (31), and weight gain (39).

Epigenetic processes (ie. DNA methylation) may provide a mechanism for the regulation of gene expression in response to lipid and/or could help explain the differential responses in gene expression in the skeletal muscle of lean vs. severely obese individuals. The extent to which methylation changed in response to lipid oversupply tended to be lower in the obese subjects (Fig. 4). This finding is in accordance with previous work showing that individuals at a greater risk of developing metabolic disease tended to have a lower sensitivity to environmental challenges (ie. high-fat feeding) in terms of the ability to regulate changes in DNA methylation (45). With lipid oversupply we observed significant increases in *PPARδ* methylation at two

cytosines positioned within 100bp of the TSS in the lean, however, there were no significant changes in the methylation status of these two cytosines in the obese (Fig. 4). A cluster of cytosines -78 to -61bp relative to the TSS increased in the lean in response to lipid oversupply suggesting the increase in methylation was targeted to the promoter region of *PPARδ* (Fig. 4). Of the cytosines within the cluster, the central one (-71bp from the TSS at position 6) was highly related to *PPARδ* mRNA content (Fig. 6).

DNA methylation is generally accepted to regulate gene transcription by directly impeding the binding of transcriptional factors to their target sites and through the recruitment of methyl-binding proteins (21). However, our findings indicate a positive relationship between the extent of *PPARδ* promoter methylation and *PPARδ* mRNA content in response to lipid oversupply (Fig. 6). In support of our findings, in human skeletal muscle Barres et al. (46) identified a subset of genes with positive relationships between gene expression and promoter methylation and suggested that DNA methylation at a transcriptional repressor binding site could subsequently induce gene expression. Pipaon et al. (47) found that increased methylation of *p73*, a gene related to the p53 tumor suppressor protein, blocked the binding of the zinc finger transcription factor repressor protein ZEB in human fibroblasts, in turn promoting the expression of *p73*; treatment with an inhibitor of DNA methyltransferase significantly also reduced the expression levels of *p73* (47). Similarly, Ando et al.(48) found that demethylation of a repressor binding site elicited a concomitant decrease in gene expression (48). Although speculative at this point, our findings indicate a potential mechanism regulating the lipid-induced increase in *PPARδ* gene expression in the lean could be the blockage of a repressive factor from binding to the *PPARδ* promoter via DNA methylation.

In the present study there were no baseline differences in *PGC-1α* and *PDK4* mRNA content and promoter methylation between the lean and severely obese women which is inconsistent with a recent report (46) where mRNA content of *PGC-1a* was significantly lower and *PDK4* mRNA content was significantly higher in muscle biopsies obtained from obese subjects. A possible reason for these divergent findings is that the current study utilized HSkMC, where hormonal and/or neural input that could differ with obesity and potentially influence gene expression are eliminated. In terms of promoter methylation, in Barres et al. (46) the majority of methylated cytosines in the *PGC-1α* and *PDK4* promoter regions were found within non-CpG sites whereas the present study only examined CpG sites, which have been previously demonstrated to regulate gene expression (22). Additionally, the composition of lipid incubation media has differed between studies; an increase in *PGC-1a* promoter methylation was evident with a 48hr 500μM palmitate incubation whereas we did not observed changes with a 48hr 250μM 1:1 oleate:palmitate lipid mixture. It is well-known that different fatty acids impart specific and unique effects, or even opposing actions, on cellular functions (32), which likely explains why findings differ between studies. We chose to utilize the oleate:palmitate mixture as it more closely mimics physiological conditions (32).

In summary, primary human skeletal muscle cell cultures (HSkMC) were utilized to study adaptations to a lipid stimulus in the skeletal muscle of lean and severely obese humans. Our findings indicate a coordinated lipid-induced activation of genes linked with FAO and mitochondrial content among lean individuals in response to lipid oversupply that is largely absent with obesity. In HSkMC from severely obese individuals, *PPARδ* displayed differential methylation patterns in the promoter region compared to cells derived from lean subjects; changes in the methylation signature may thus play a role in controlling gene expression.

GRANTS

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

DISCLOSURES

The authors have nothing to declare.

REFERENCES

1. Kelley DE, Mandarino LJ**.** Fuel selection in human skeletal muscle in insulin resistance: a reexamination. Diabetes. 2000;49(5):677-83.

2. Boyle KE, Canham JP, Consitt LA, Zheng D, Koves TR, Gavin TP, et al. A high-fat diet elicits differential responses in genes coordinating oxidative metabolism in skeletal muscle of lean and obese individuals. J Clin Endocrinol Metab. 2011;96(3):775-81. Epub 2010 Dec 29.

3. Battaglia GM, Zheng D, Hickner RC, Houmard JA**.** Effect of exercise training on metabolic flexibility in response to a high-fat diet in obese individuals. Am J Physiol Endocrinol Metab. 2012;303(12):E1440-5. doi: 10.152/ajpendo.00355.2012. Epub 2012 Oct 9.

4. Boyle KE, Zheng D, Anderson EJ, Neufer PD, Houmard JA**.** Mitochondrial lipid oxidation is impaired in cultured myotubes from obese humans. Int J Obes. 2011;25(10):201.

5. Cameron-Smith D, Burke LM, Angus DJ, Tunstall RJ, Cox GR, Bonen A, et al. A short-term, high-fat diet up-regulates lipid metabolism and gene expression in human skeletal muscle. Am J Clin Nutr. 2003;77(2):313-8.

6. Crunkhorn S, Dearie F, Mantzoros C, Gami H, da Silva WS, Espinoza D, et al. Peroxisome proliferator activator receptor gamma coactivator-1 expression is reduced in obesity: potential pathogenic role of saturated fatty acids and p38 mitogen-activated protein kinase activation. J Biol Chem. 2007;282(21):15439-50. Epub 2007 Apr 6.

7. Hancock CR, Han DH, Chen M, Terada S, Yasuda T, Wright DC, et al. High-fat diets cause insulin resistance despite an increase in muscle mitochondria. Proc Natl Acad Sci U S A. 2008;105(22):7815-20. Epub 2008 May 28.

8. Hock MB, Kralli A**.** Transcriptional control of mitochondrial biogenesis and function. Annu Rev Physiol. 2009;71:177-203.

9. Consitt LA, Bell JA, Koves TR, Muoio DM, Hulver MW, Haynie KR, et al. Peroxisome proliferator-activated receptor-gamma coactivator-1alpha overexpression increases lipid oxidation in myocytes from extremely obese individuals. Diabetes. 2010;59(6):1407-15. Epub 2010 Mar 3.

10. Zurlo F, Lillioja S, Esposito-Del Puente A, Nyomba BL, Raz I, Saad MF, et al. Low ratio of fat to carbohydrate oxidation as predictor of weight gain: study of 24-h RQ. Am J Physiol. 1990;259(5 Pt 1):E650-7.

11. Galgani JE, Moro C, Ravussin E**.** Metabolic flexibility and insulin resistance. Am J Physiol Endocrinol Metab. 2008;295(5):E1009-17. doi: 10.152/ajpendo.90558.2008. Epub 2008 Sep 2.

12. Thomas CD, Peters JC, Reed GW, Abumrad NN, Sun M, Hill JO**.** Nutrient balance and energy expenditure during ad libitum feeding of high-fat and high-carbohydrate diets in humans. Am J Clin Nutr. 1992;55(5):934-42.

13. Jacobsen SC, Brons C, Bork-Jensen J, Ribel-Madsen R, Yang B, Lara E, et al. Effects of short-term high-fat overfeeding on genome-wide DNA methylation in the skeletal muscle of healthy young men. Diabetologia. 2012;55(12):3341-9. doi: 10.1007/s00125-012-2717-8. Epub 2012 Sep 8.

14. Barres R, Osler ME, Yan J, Rune A, Fritz T, Caidahl K, et al. Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density. Cell Metab. 2009;10(3):189-98.

15. Watt MJ, Hoy AJ, Muoio DM, Coleman RA**.** Distinct roles of specific fatty acids in cellular processes: implications for interpreting and reporting experiments. Am J Physiol Endocrinol Metab. 2012;302(1):E1-3. doi: 10.1152/ajpendo.00418.2011.

16. Evans WJ, Phinney SD, Young VR**.** Suction applied to a muscle biopsy maximizes sample size. Med Sci Sports Exerc. 1982;14(1):101-2.

17. Berggren JR, Tanner CJ, Houmard JA**.** Primary cell cultures in the study of human muscle metabolism. Exerc Sport Sci Rev. 2007;35(2):56-61.

18. Muoio DM, Way JM, Tanner CJ, Winegar DA, Kliewer SA, Houmard JA, et al. Peroxisome proliferator-activated receptor-alpha regulates fatty acid utilization in primary human skeletal muscle cells. Diabetes. 2002;51(4):901-9.

19. Sandoval J, Heyn H, Moran S, Serra-Musach J, Pujana MA, Bibikova M, et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics. 2011;6(6):692-702. Epub 2011 Jun 1.

20. Astrup A, Buemann B, Christensen NJ, Toubro S**.** Failure to increase lipid oxidation in response to increasing dietary fat content in formerly obese women. Am J Physiol. 1994;266(4 Pt 1):E592-9.

21. Kelley DE**.** Skeletal muscle fat oxidation: timing and flexibility are everything. J Clin Invest. 2005;115(7):1699-702.

22. Ravussin E**.** Metabolic differences and the development of obesity. Metabolism. 1995;44(9 Suppl 3):12-4.

23. Kelly DP, Scarpulla RC**.** Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev. 2004;18(4):357-68.

24. Muoio DM, MacLean PS, Lang DB, Li S, Houmard JA, Way JM, et al. Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) alpha knock-out mice. Evidence for compensatory regulation by PPAR delta. J Biol Chem. 2002;277(29):26089-97. Epub 2002 May 6.

25. Lee CH, Olson P, Evans RM**.** Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. Endocrinology. 2003;144(6):2201-7.

26. Muoio DM, Koves TR**.** Skeletal muscle adaptation to fatty acid depends on coordinated actions of the PPARs and PGC1 alpha: implications for metabolic disease. Appl Physiol Nutr Metab. 2007;32(5):874-83.

27. Wagner KD, Wagner N**.** Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) acts as regulator of metabolism linked to multiple cellular functions. Pharmacol Ther. 2010;125(3):423-35. doi: 10.1016/j.pharmthera.2009.12.001. Epub Dec 22.

28. Sugden MC, Holness MJ**.** Mechanisms underlying regulation of the expression and activities of the mammalian pyruvate dehydrogenase kinases. Arch Physiol Biochem. 2006;112(3):139-49.

29. Bergouignan A, Gozansky WS, Barry DW, Leitner W, MacLean PS, Hill JO, et al. Increasing dietary fat elicits similar changes in fat oxidation and markers of muscle oxidative capacity in lean and obese humans. PLoS One. 2012;7(1):e30164. doi: 10.1371/journal.pone.0030164. Epub 2012 Jan 12.

30. Hulver MW, Berggren JR, Cortright RN, Dudek RW, Thompson RP, Pories WJ, et al. Skeletal muscle lipid metabolism with obesity. Am J Physiol Endocrinol Metab. 2003;284(4):E741-7. Epub 2002 Dec 27.

31. Huang B, Wu P, Bowker-Kinley MM, Harris RA**.** Regulation of pyruvate dehydrogenase kinase expression by peroxisome proliferator-activated receptor-alpha ligands, glucocorticoids, and insulin. Diabetes. 2002;51(2):276-83.

32. Brons C, Jacobsen S, Nilsson E, Ronn T, Jensen CB, Storgaard H, et al. Deoxyribonucleic acid methylation and gene expression of PPARGC1A in human muscle is influenced by high-fat overfeeding in a birth-weight-dependent manner. J Clin Endocrinol Metab. 2010;95(6):3048-56. doi: 10.1210/jc.2009-413. Epub 10 Apr 21.

33. Miranda TB, Jones PA**.** DNA methylation: the nuts and bolts of repression. J Cell Physiol. 2007;213(2):384-90.

34. Barres R, Kirchner H, Rasmussen M, Yan J, Kantor FR, Krook A, et al. Weight Loss after Gastric Bypass Surgery in Human Obesity Remodels Promoter Methylation. Cell Rep. 2013;10(13):00125-3.

35. Pipaon C, Real PJ, Fernandez-Luna JL**.** Defective binding of transcriptional repressor ZEB via DNA methylation contributes to increased constitutive levels of p73 in Fanconi anemia cells. FEBS Lett. 2005;579(21):4610-4.

36. Ando T, Nishimura M, Oka Y**.** Decitabine (5-Aza-2'-deoxycytidine) decreased DNA methylation and expression of MDR-1 gene in K562/ADM cells. Leukemia. 2000;14(11):1915-20.

37. Vaissiere T, Sawan C, Herceg Z**.** Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat Res. 2008;659(1-2):40-8. Epub 2008 Feb 29.

TABLES AND FIGURES

Table 2.1. Subject characteristics**.**

	Lean (n=12)	**Obese (n=10)**
Age (y)	23.4±1.5	30.2±2.6*
Stature (cm)	164.7±1.8	165.5±2.2
Mass (kg)	62.6±1.3	113.7±6.3*
BMI (kg/m^2)	22.8±0.7	41.3±1.5*
Fasting glucose (mmol/L)	4.6±0.1	4.9±0.1
Fasting insulin (uU/L)	4.3±1.2	17.3±5.0*
HOMA-IR	0.9±0.3	3.8±1.1*
Plasma cholesterol (mg/dl)	179±8.2	171±10.1
Plasma triglycerides (mg/dl)	102±7.4	121±21.0
HDL (mg/dl)	56±4.0	40±3.4*

Results are expressed as mean±SEM

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

Table 2.2. Genes regulated by the NRFs and PPARs.

	Response Element		
Gene	**NRF**	**PPAR**	**Description**
ANGTPL4		X	angiopoietin-like 4
CS		X	citrate synthase
PDK4		X	pyruvate dehydrogenase kinase 4
UCP3		X	mitochondrial uncoupling protein 3
COX6C	X		cytochrome c oxidase subunit 6 C
CYCS	X		somatic cytochrome c
GFM1	X		mitochondrial elongation factor G 1
MRLP2	X		mitochondrial ribosomal protein L 2
TFAM	X		mitochondrial transcription factor A
TFB2M	X		mitochondrial transcription factor B 2

Figure 2.1. Relative change in mRNA content in response to 48hr 250µM oleate:palmitate treatment in cultured myotubes (HSkMC) from lean (open bars) and severely obese (solid bars) donors. Data are expressed as the fold change (lipid-treated divided by baseline) (mean± SEM). No change is a value of 1, which is represented by the dashed line, with values > 1.0 indicative of an increase in respective mRNA content with the lipid-treatment. *, Significant difference ($P \leq 0.05$) between lean and obese individuals.

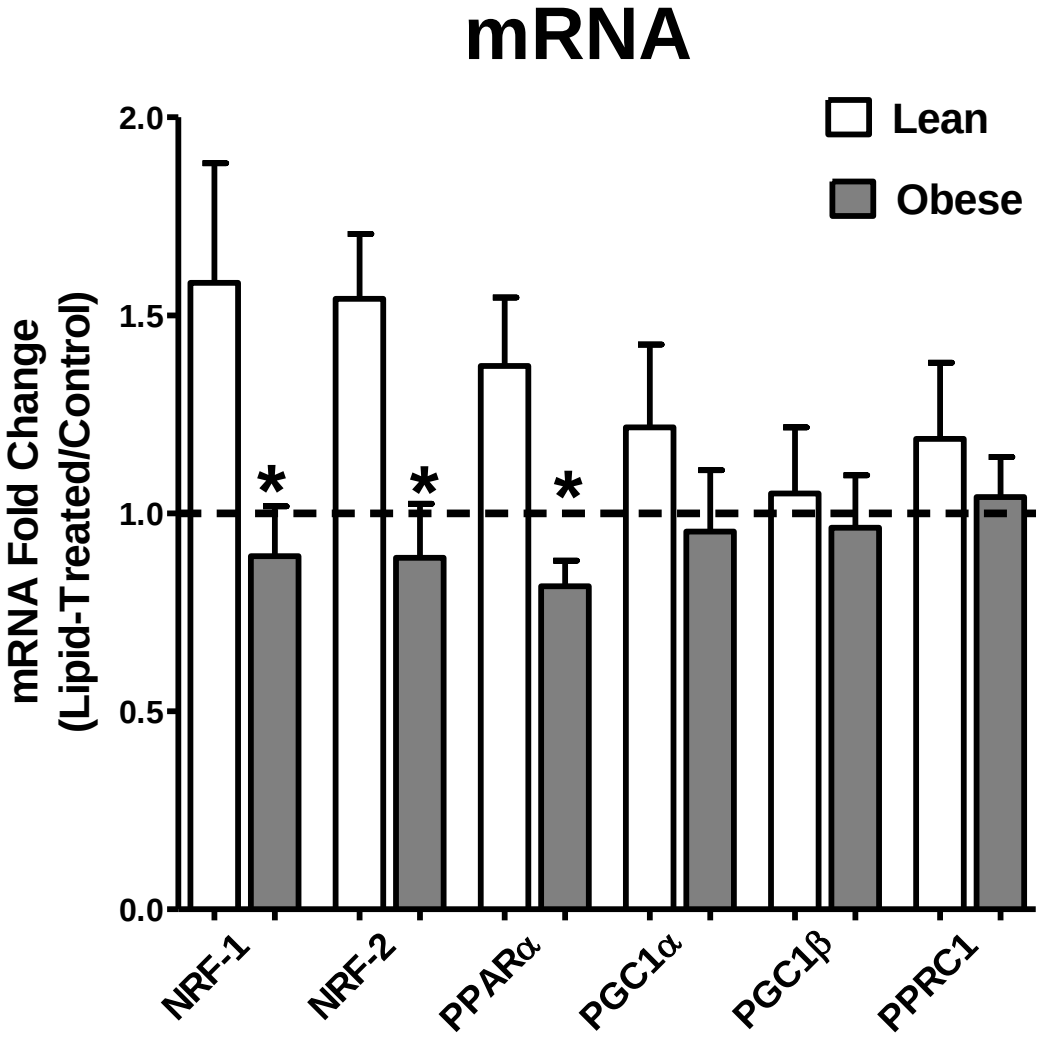


Figure 2.2. Effect of lipid oversupply (48hr 250µM oleate:palmitate treatment) on mRNA content of *PPARδ* in cultured myotubes (HSkMC) from lean (open bars) and severely obese (solid bars) donors. Data are expressed as mean±SEM. *, Significant difference ($P \leq 0.05$) between lean and obese individuals. Φ, Significant difference ($P \leq 0.05$) between control and lipid-treated.

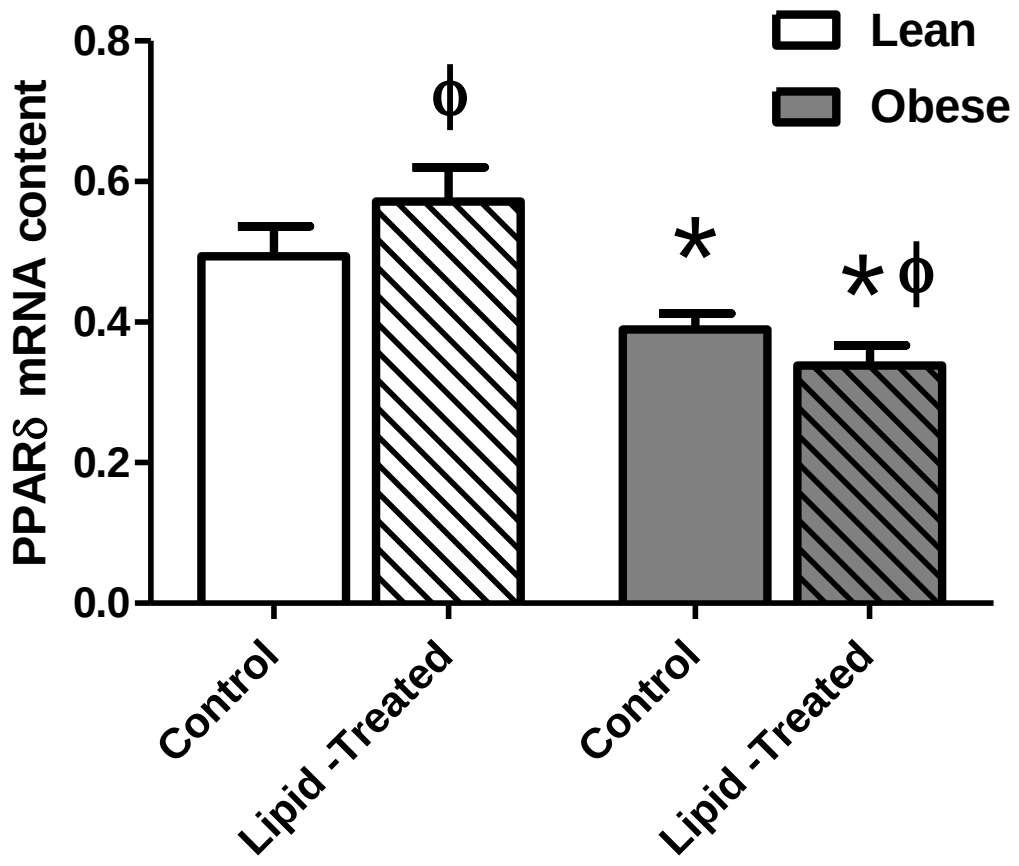
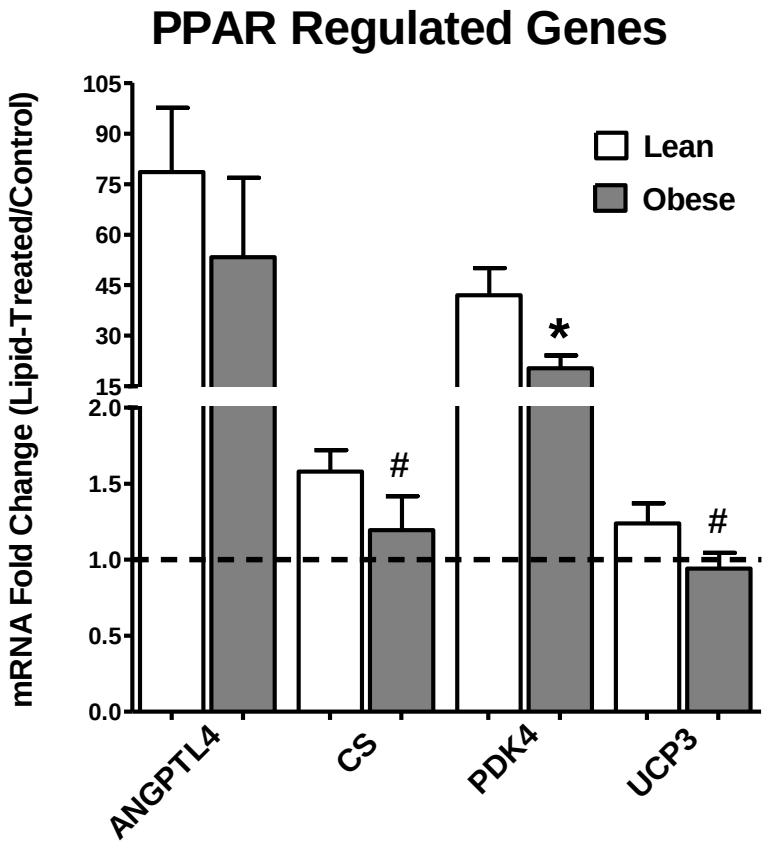


Figure 2.3. Relative change in the mRNA content of PPAR-regulated genes (A) and NRF-regulated genes (B) in response to 48hr 250μM oleate:palmitate treatment in cultured myotubes (HSkMC) from lean (open bars) and severely obese (solid bars) donors. Data are expressed as the fold change (lipid-treated divided by baseline) (mean± SEM). No change is a value of 1, which is represented by the dashed line, with values > 1.0 indicative of an increase in respective mRNA content with the lipid-treatment. *, Significant difference ($P \leq 0.05$) between lean and obese individuals. #, Difference between lean and obese individuals approached significance ($P \leq 0.1$).

A


PPAR Regulated Genes
mRNA Fold Change (Lipid-Treated/Control)
105
90
75
60
45
30
15
2.0
1.5
1.0
0.5
0.0
Lean
Obese
*
#
#
ANGPTL4
CS
PDK4
UCP3

B

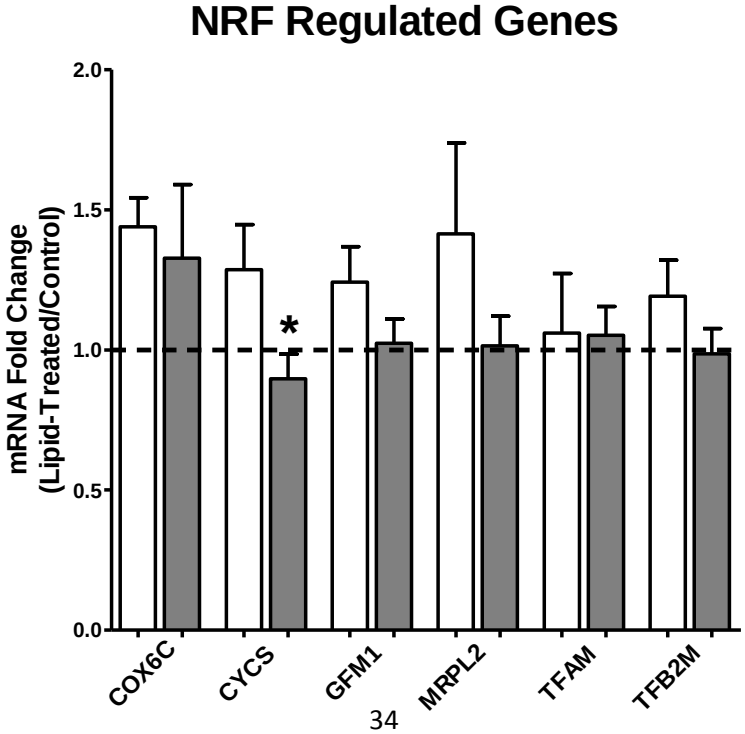
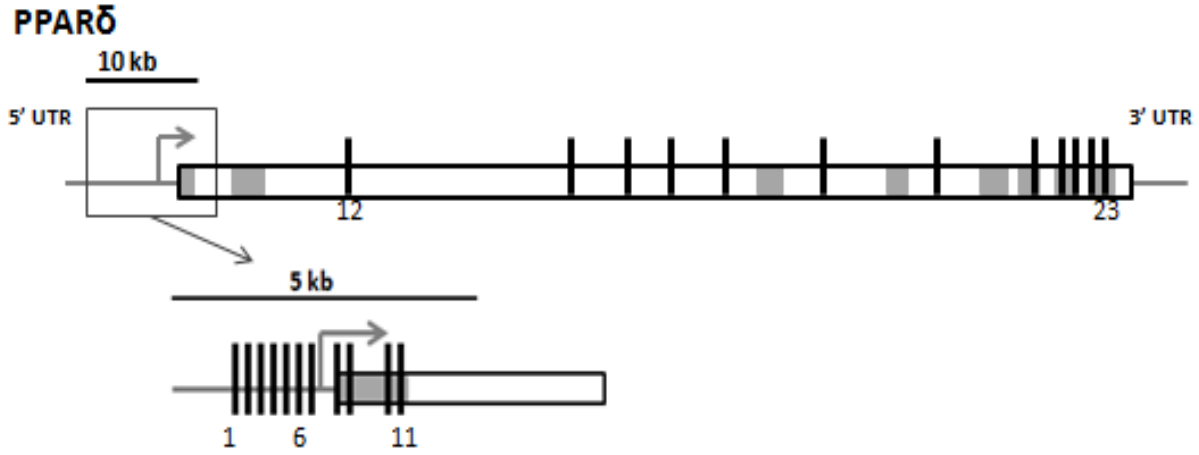
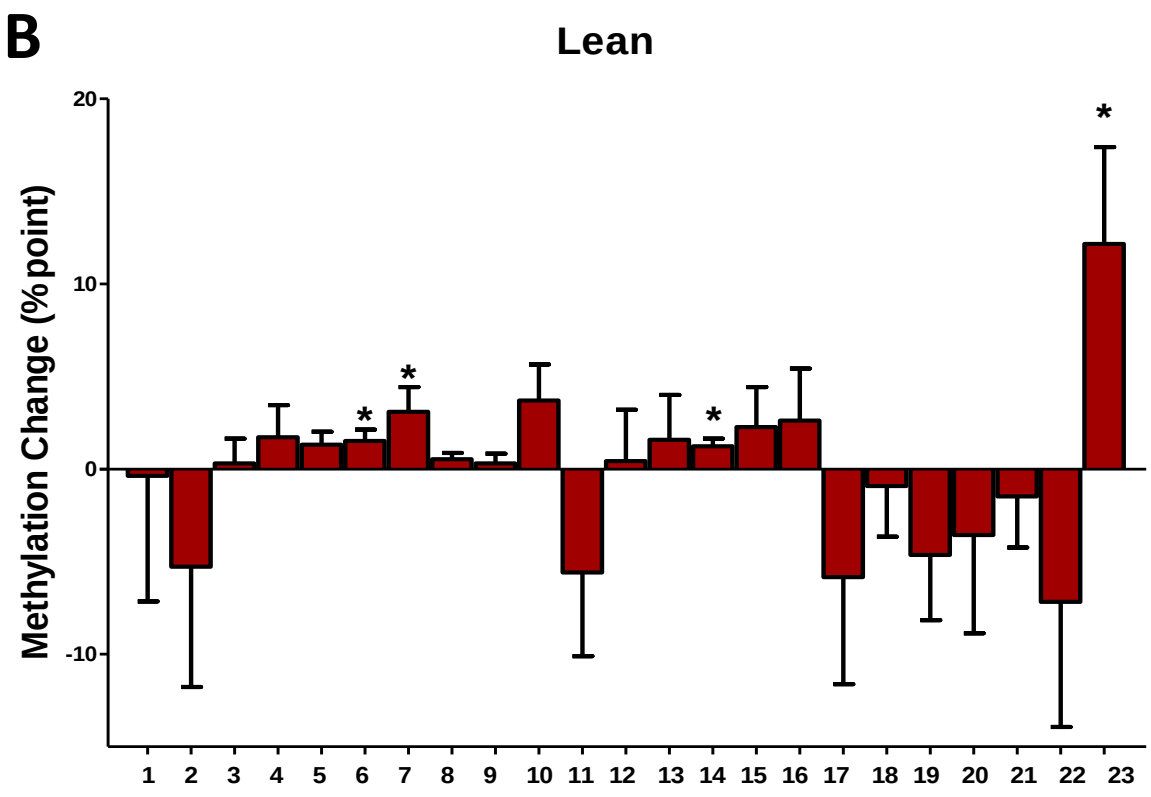

NRF Regulated Genes
mRNA Fold Change (Lipid-Treated/Control)
2.0
1.5
1.0
0.5
0.0
*
COX6C
CYCS
GFM1
MRPL2
TFAM
TFB2M

Figure 2.4. *PPARδ* gene methylation. Visualization of the 23 measured CpG sites within the *PPARδ* gene (A). DNA methylation changes in response to lipid oversupply (methylation value in the lipid-treated condition minus the baseline methylation value) at each of the 23 measured CpG sites among the lean individuals (B) and the severely obese individuals (C). *, Significant difference ($P \leq 0.05$) between the baseline and lipid-treated condition. TSS is indicated by the arrow.

A




B
Lean
Methylation Change (%point)
20
10
0
-10
*
*
*
*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

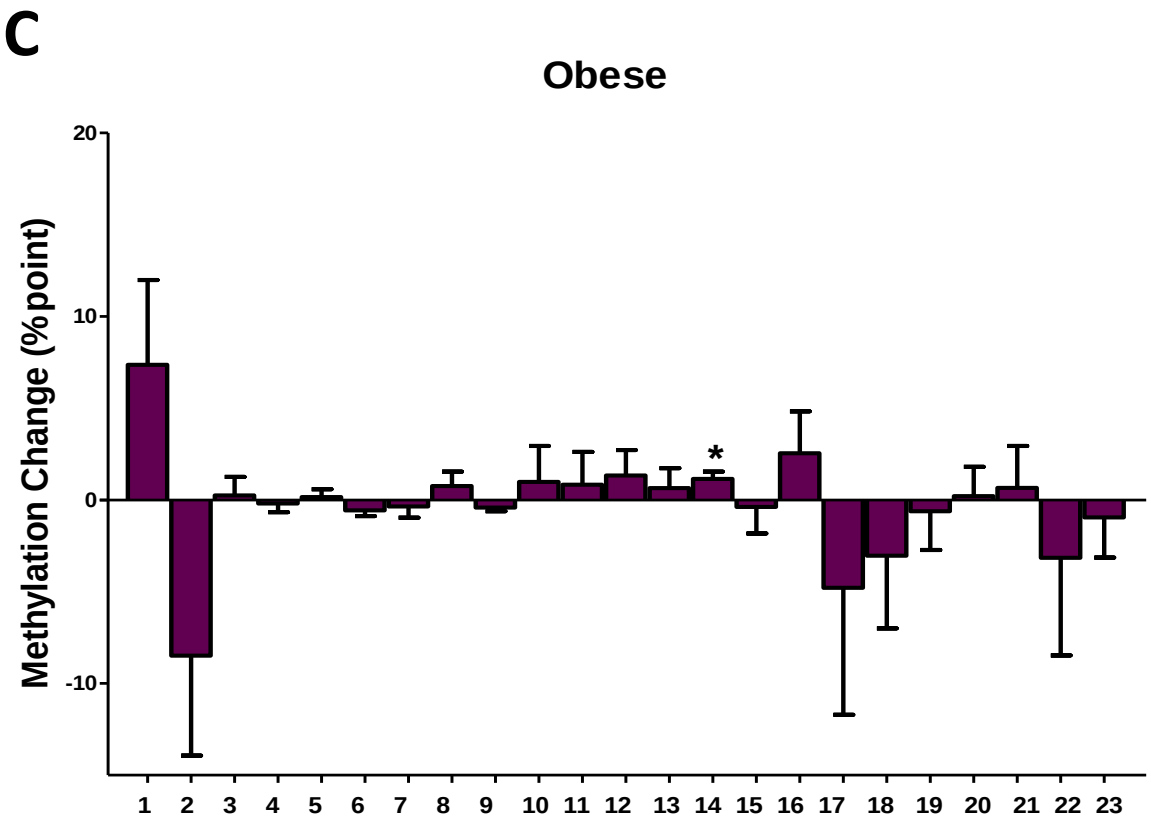

C
Obese
Methylation Change (%point)
20
10
0
-10
*
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

Figure 2.5. Methylation of 23 cytosines within the *PPARδ* gene in the lipid-treated condition. Data are presented as obese methylation values relative to lean (the obese lipid-treated methylation value divided by the lean lipid-treated methylation) (mean± SEM). No differences in the methylation of cytosines in the lipid-treated condition between lean and obese is a value of 1, which is represented by the dashed line, with values < 1.0 indicative of lower methylation levels among the obese compared to the lean.*, Significant difference ($P \leq 0.05$) between lean and obese individuals. #, Difference between lean and obese individuals approached significance ($P \leq 0.1$).

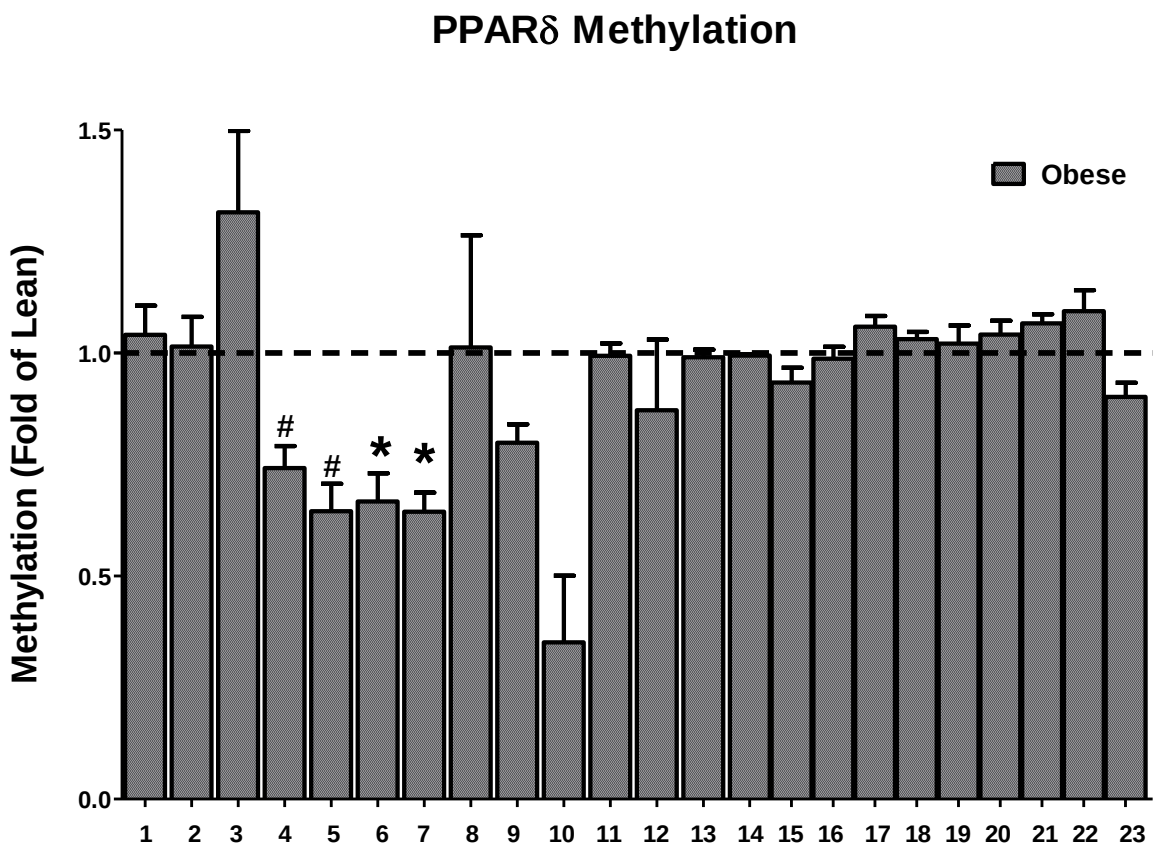
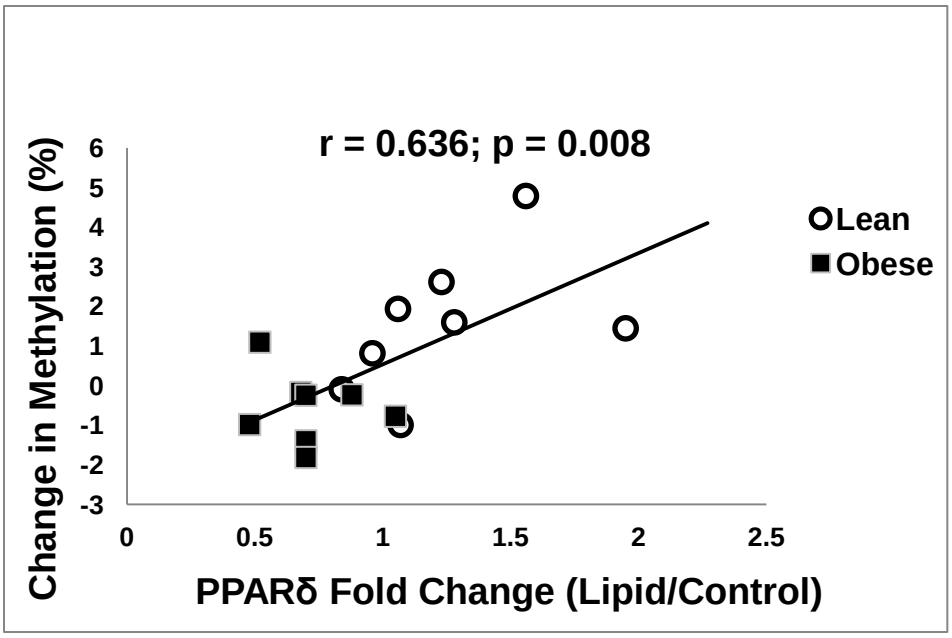
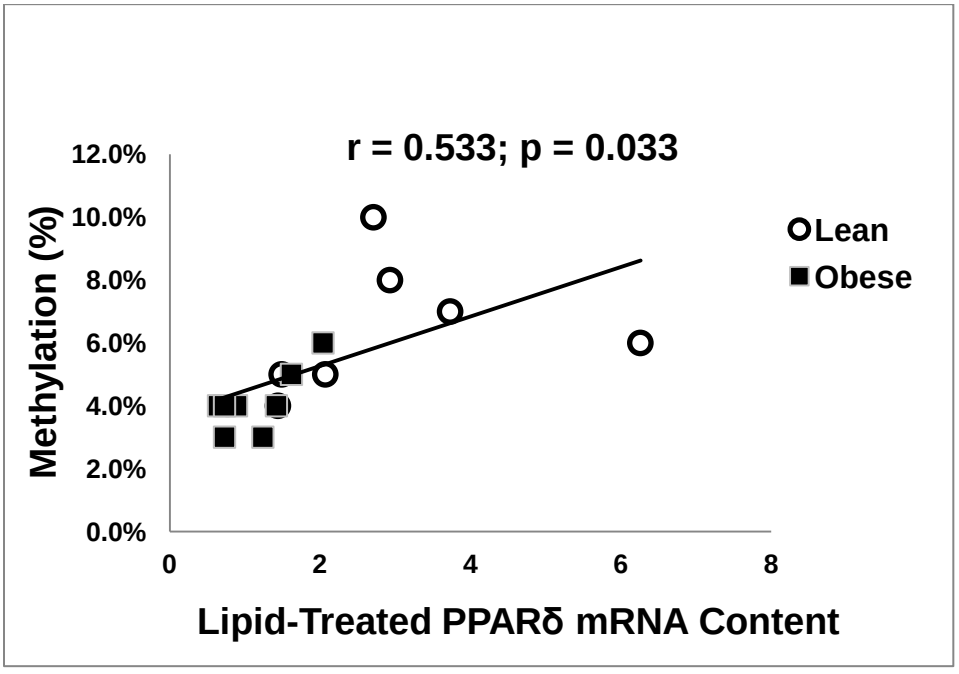


Figure 2.6. The association of PPARδ methylation and *PPARδ* mRNA content. The change in methylation in response to lipid oversupply (methylation value in the lipid-treated condition minus the baseline methylation value) of the cytosine at position 6 was positively related to the relative change in *PPARδ* mRNA content (lipid-treated divided by baseline) (A). Methylation of the cytosine at position 6 in the lipid-treated condition was positively related to *PPARδ* mRNA content in the lipid-treated condition (B).

A


r = 0.636; p = 0.008
Change in Methylation (%)
6
5
4
3
2
1
0
-1
-2
-3
0
0.5
1
1.5
2
2.5
PPARδ Fold Change (Lipid/Control)
Lean
Obese

B


r = 0.533; p = 0.033
Methylation (%)
12.0%
10.0%
8.0%
6.0%
4.0%
2.0%
0.0%
0
2
4
6
8
Lipid-Treated PPARδ mRNA Content
Lean
Obese

Supplementary Table 2.1. Baseline and lipid-treated methylation values for 23 CpG sites within the *PPARδ* gene.

Target ID	Human Genome Map Position	Position	Distance from TSS	Lean Control			Lean Lipid-Treated			Obese Control			Obese Lipid-Treated		
				N	Mean (%)	SEM (%)	N	Mean (%)	SEM (%)	N	Mean (%)	SEM (%)	N	Mean (%)	SEM (%)
cg21748751	35309867	1	-468	9	60.5	2.3	9	60.1	6.4	9	58.0	3.1	9	62.6	3.9
cg18204017	35309928	2	-407	9	90.3	1.9	8	84.6	5.8	9	92.8	0.8	9	85.9	5.6
cg00657095	35310123	3	-212	9	4.9	0.6	6	4.6	0.6	9	5.4	0.7	9	5.7	0.8
cg17499041	35310257	4	-78	9	12.3	1.1	8	13.1	1.6	9	10.6	0.8	8	9.71#	0.6
cg06610850	35310260	5	-75	9	3.3	0.3	8	4.4	0.7	9	2.57#	0.3	8	2.84#	0.3
cg05528533	35310264	6	-71	9	5.1	0.3	8	6.4	0.7	9	5.1	0.5	8	4.25*	0.4
cg08893449	35310274	7	-61	9	7.0	0.3	8	9.7	1.3	9	7.3	0.5	8	6.27*	0.4
cg02943769	35310542	8	207	9	2.7	0.2	9	3.3	0.4	9	2.6	0.2	9	3.3	0.8
cg15729095	35310685	9	350	9	4.0	0.3	9	4.3	0.5	9	3.8	0.1	8	3.4	0.2
cg26668919	35311534	10	1199	9	6.5	2.4	8	10.7	3.7	7	3.4	0.4	7	3.8	1.6
cg11071407	35312842	11	2507	9	79.6	1.7	9	74.0	4.5	9	74.12#	2.3	9	73.5	2.0
cg14015015	35327915	12	17580	9	7.4	1.7	8	7.8	1.3	9	5.5	0.3	9	6.8	1.2
cg25145023	35348742	13	38407	9	86.4	1.7	9	88.0	1.7	9	86.5	1.0	9	87.2	1.5
cg01519223	35351379	14	41044	9	95.2	0.4	9	96.5	0.5	9	94.8	0.3	9	95.9	0.3
cg26774385	35354049	15	43714	9	87.2	1.9	9	89.4	2.1	9	84.5	2.5	9	83.5	3.0
cg01101911	35360715	16	50380	9	80.4	2.2	9	83.1	3.3	9	78.8	1.2	9	82.0	2.2
cg23903774	35370123	17	59788	9	90.8	1.2	9	84.9	5.8	9	88.2	1.4	9	90.0	2.1
cg15611037	35387204	18	76869	9	87.0	2.2	9	86.1	1.9	9	88.2	0.9	9	88.8	1.4
cg06029061	35392246	19	81911	9	83.2	2.3	9	78.6	4.7	9	80.9	1.9	9	80.2	3.2
cg23318156	35392306	20	81971	9	84.8	2.8	9	81.2	4.5	9	84.1	1.9	9	84.6	2.5
cg03772072	35392381	21	82046	9	89.8	4.5	9	88.3	3.5	9	92.9	0.8	9	94.1	1.8
cg17442907	35392736	22	82401	9	82.5	2.0	9	75.3	6.6	9	80.6	2.0	9	82.4	3.5
cg05544828	35395209	23	84874	9	59.7	4.0	9	71.8	3.9	9	63.9	2.5	9	64.8	2.3

Lean vs. obese *$P \leq 0.05$
Lean vs. obese #$P \leq 0.10$

CHAPTER 3: EPIGENETIC MODIFICATIONS PLAY A ROLE IN THE DIFFERENTIAL TRANSCRIPTIONAL REGULATION OF *CPT1B* IN LEAN AND SEVERELY OBESE WOMEN IN RESPONSE TO LIPID OVERSUPPLY

ABSTRACT

The skeletal muscle of severely obese individuals cannot increase fatty acid oxidation (FAO) in response to dietary lipid, which is associated with a failure to coordinately upregulate genes involved in FAO, termed metabolic *in*flexibility. While the molecular mechanisms contributing to this metabolic *in*flexibility are not evident, a possible candidate is carnitine palmitoyltransferase 1B (CPT1B), which is a rate-limiting step in FAO. The present study was undertaken to determine if the differential response to lipid oversupply of the *CPT1B* gene in skeletal muscle with severe obesity is linked to epigenetic modifications (ie. DNA methylation and histone acetylation) that impact transcriptional activation. In primary human skeletal muscle cultures the expression of *CPT1B* was blunted in severely obese women compared to their lean counterparts in response to lipid oversupply, which was accompanied by changes in CpG methylation, H3/H4 histone acetylation, and PPARδ and hepatocyte nuclear factor 4α (HNF-4α) transcription factor binding to the *CPT1B* promoter. Our findings shed new light on the epigenetic modifications that play important roles in the transcriptional upregulation of *CPT1B* in response to a physiologically relevant lipid mixture in human skeletal muscle, which is a major site of fatty acid catabolism. In terms of identifying mechanisms that contribute to the metabolic *in*flexibility with severe obesity, it is likely that differential DNA methylation partially explains the depressed expression of *CPT1B* among the obese women in response to lipid oversupply.

INTRODUCTION

Mounting evidence indicates that metabolic diseases (i.e. obesity, type 2 diabetes, insulin resistance) are associated with an inability to oxidize lipids (1) and adjust substrate oxidation according to nutrient availability, termed 'metabolic flexibility' (2). These impairments are particularly evident in the severely obese (Class III; BMI > 40kg/m^2) where there is an impaired ability to oxidize lipid (fatty acid oxidation, FAO) in skeletal muscle and an inability to increase skeletal muscle FAO in response to a 3-5d high-fat diet (3, 4). These decrements likely contribute to intramuscular lipid accumulation associated with insulin resistance, weight gain (5), and weight regain after weight loss (6).

The molecular mechanisms contributing to this inability to oxidize lipid with severe obesity, however, are not evident. A possible candidate is carnitine palmitoyltransferase (CPT1), which mediates the transfer of long chain fatty acids across the outer mitochondrial membrane and is a rate-limiting step in FAO (7). The activity of the muscle-type CPT1 (encoded by the *CPT1B* gene) is reduced in skeletal muscle with obesity, contributing to a decrease in FAO (4). In relation to metabolic flexibility, in the skeletal muscle of lean subjects a high-fat diet increased the expression of *CTP1B* and the peroxisome proliferator-activated receptors (*PPAR*s) which are upstream regulators of *CPT1B* expression (8). Conversely, a high-fat diet resulted in little to no change in the expression of these genes in the skeletal muscle of severely obese subjects (8). These data indicate a differential response to lipid oversupply with obesity which could contribute to positive lipid balance and ectopic lipid accumulation.

Acute epigenetic modifications of the genome, such as DNA methylation and histone acetylation, may provide a connection between nutritional factors, gene expression, and

metabolic health. Histone acetylation affects both chromatin structure as well as the interaction of transcription-regulatory proteins with target DNA in chromatin (9). For example, an increase in the acetyl groups on histones will result in an open chromatin structure to facilitate accessibility of transcriptional machinery to DNA templates in chromatin, in turn increasing the expression of a gene. Conversely, histone *de*acetylation may induce other epigenetic modifications (i.e. DNA methylation) leading to a decrease in gene expression. There is a large body of evidence on the functional significance of both histone acetylation and DNA methylation levels and their correlation with gene expression as well as their importance in integrating environmental stimuli, such as diet, in the control of gene expression (9, 10).

CPT1B is the predominant CPT1 isoform expressed in skeletal muscle (7). While *CPT1B* plays an important role in human skeletal muscle lipid metabolism *and* has been shown to be differentially regulated in the skeletal muscle of severely obese individuals in response to a high-fat diet (8), the transcriptional regulation of this gene in response to lipid oversupply has not been extensively examined. The purpose of the present study was to determine if the differential response to lipid oversupply of the *CPT1B* gene in skeletal muscle with severe obesity is linked to epigenetic modifications (i.e. DNA methylation and histone acetylation) that impact transcriptional activation. To our knowledge this is the first study to examine the transcriptional regulation of *CPT1B* in response to a physiologically relevant lipid oversupply, in terms of concentration and ratios of saturated to unsaturated fatty acids, in primary human skeletal muscle cell cultures (HSkMC). By utilizing HSkMC we were able to study the molecular adaptations to a lipid stimulus in an environment void of *in-vivo* hormonal and neural stimuli and thus intrinsic to skeletal muscle itself.

EXPERIMENTAL PROCEDURES

Study Design

Briefly, the design of the study was to compare the mRNA response of the rate-limiting enzyme *CPT1B* to lipid oversupply in lean vs. severely obese subjects. Skeletal muscle was obtained from the vastus lateralis and used to derive primary human skeletal muscle cell cultures (HSkMC). After differentiation into myotubes, HSkMC were incubated in a physiologically relevant lipid mixture (250µM oleate:palmitate) for 48hr and mRNA content, DNA methylation, histone acetylation, and transcription factor binding to the *CPT1B* promoter region were determined.

Materials

All chemical reagents and substrates were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. Dulbecco's Phosphate-Buffered Saline (DPBS), fetal bovine serum, heat-inactivated horse serum, gentamicin, 0.05% trypsin EDTA, and Hanks's balanced salt solution were obtained from Invitrogen. Growth media and differentiation media consisted of low glucose (5 mmol/L) Dulbecco's Modified Eagles Medium from Invitrogen. Type I collagen-coated tissue culture plates were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). PCR reagents were purchased from Applied Biosystems (Foster City, CA, USA).

Human Subjects

Muscle biopsies were obtained using the percutaneous needle biopsy technique (11) under local anesthesia (0.01% lidocaine) from the vastus lateralis of 9 lean (BMI= 22.8 kg/m^2 ±0.7; Age= 23.4yrs ±1.5) and 9 severely obese (BMI= 41.3 kg/m^2 ±1.5; Age= 29.9yrs±2.9)

Caucasian women. Characteristics are presented in Table 1. Participants were relatively young, free from disease, nonsmokers and not taking medications known to alter metabolism. All procedures were approved by the East Carolina University Institutional Board.

Primary HSkMC

Satellite cells were isolated from ~50-100mg of fresh muscle tissue and cultured into myoblasts as previously described (12). For experiments cells were sub-cultured into T-150 flasks and 10cm dishes. Upon reaching ~80-90% confluence, differentiation was induced by switching the growth media to low-serum differentiation media containing 2% heat-inactivated horse serum, 0.05 mg/ml fetuin, and 5 µg/ml gentamicin. On day 5 of differentiation, myotubes were given fresh differentiation media supplemented with 1) 0.1% bovine serum albumin (BSA) + 1mM carnitine (CONTROL) or 2) 250µM oleate:palmitate (1:1 ratio) bound to 0.1% BSA + 1mM carnitine (LIPID) for a total incubation period of 48 hours. Myotubes were harvested on day seven similar to previous work (13). There were no obvious differences in the extent of myotube morphology or differentiation between lean and obese HSkMC.

RNA Isolation and mRNA quantification

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) with on-column DNase digestion using the RNase-Free DNase Set (Qiagen, Valencia, CA) to remove residual DNA. RNA was quantified using the NanoDrop 1000 Spectrophotometer Version 3.7.1 from Thermoscientific (Wilmington, DE, USA) and concentration was determined by measuring the absorbance at 260nm. 2ug of RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA, USA). PCR was performed in triplicate using the Applied Biosystems ABI 7900HT sequence detection

instrument and software with Taqman Universal PCR Master Mix and TaqMan gene expression assays (Applied Biosystems, Foster City, California) in accordance with manufacturer's instructions. Using standard techniques, reactions were run with the following thermal cycling conditions: 50˚C for 2 min; 95˚C for 10 min; and 40 cycles of 95˚C for 15 s; followed by 60˚C for 1 min. mRNA content was measured using the comparative Ct method with a multiplexed endogenous control (18S) and converted to a linear function by using a base 2 antilog transformation.

DNA Isolation

Cells were washed with DPBS and trypsinized with trypsin-EDTA (0.05% trypsin and 0.25% EDTA). Total DNA (mitochondrial and nuclea) was extracted from cells using a QIAamp DNA mini kit (Qiagen, Valencia, CA) and total DNA quantified using the NanoDrop 1000 Spectrophotometer Version 3.7.1 from Thermoscientific (Wilmington, DE, USA).

Bisulfite Conversion and DNA Methylation Profiling

500ng of DNA was used to perform bisulfate conversion using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) following the manufacturer's protocol, with the alternative incubation conditions recommended when using the Illumina Infunium Methylation Assay. Genome-wide DNA methylation analysis was conducted on bisulfate treated DNA samples using the Illumina Infinium HumanMethylation 450K BeadChip, which allows the quantitative monitoring of 485,764 cytosine positions (14). 12uL of each bisulfate-converted sample was amplified and fragmented, following the manufacturer's protocol, hybridized to arrays in a balanced design and scanned on an Illumina iScan System. Data were output and analyzed using Illumina's Genome Studio software.

Chromatin Immunoprecipitation (ChIP) Assay

Cellular chromatin was cross-linked by adding 1% formaldyhyde. The cross-linking reaction was stopped by adding 0.125M glycine and cells were then scrapped from the cell culture dishes, washed with PBS, centrifuged, and resuspended in lysis buffer (5mM Pipes [pH 8.0], 85mM KCl, 0.4% NP40, Complete Mini EDTA-free protease inhibitor cocktail tablet [Roche, Branchburg, NJ]). Pelleted nuclei were resuspended in a sonication solution (50mM Tris-HCl [pH 8.1], 1% SDS, 10mM EDTA, Complete Mini protease inhibitor cocktail tablet [Roche, Branchburg, NJ]) and sheared by sonication to an average size of 1 kb. The sonicated chromatin was centrifuged and resuspended in IP buffer (16.7 mM Tris-HCl [pH 7.9], 167 mM NaCl, 0.01% SDS, 1.1% Triton-X, 1.2 mM EDTA, Complete Mini protease inhibitor cocktail tablet [Roche, Branchburg, NJ]). An aliquot of each sample was removed as "input" and used in PCR analysis. The soluble chromatin was incubated overnight at 4˚C in a rotating shaker with the following antibodies: 1) anti-PPAR alpha monoclonal (ab2779, Abcam, Cambridge, MA), 2) anti-PPAR delta polyclonal (ab125290, Abcam, Cambridge, MA), 3) anti-HNF-4 alpha monoclonal (ab41898, Abcam, Cambridge, MA), or 4) anti-acetyl-Histone H3 polyclonal (#06-599, Millipore, Billerica, MA) and anti-acetyl-Histone H4 polyclonal (#06-866, Millipore, Billerica, MA). Normal Rabbit IgG polyclonal antibody (#2729, Cell Signaling, Danvers, MA) was used as control for the ChIP assays. Immune complexes were isolated by incubation with 60 µl of ChIP-Grade Protein G Agarose Beads (Cell Signaling, Danvers, MA) for 1hr at 4°C. The complexes were serially washed in 1 ml low salt buffer (0.1% SDS, 1% Triton X-100, 0.2 M EDTA, 20 mM Tris-HCl [pH 8.1], 20% glycerol, 0.5 mM DTT, 100 mM NaCl) (twice), 1 ml of the same buffer but with high salt (500 mM NaCl) (twice), 1 ml of LiCl buffer (250 mM LiCl, 1% NP-40, 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0]) (twice), and four times with

TE (10 mM Tris-HCl [pH 7.5] and 1 mM EDTA). The complexes were eluted with two 250µl aliquots of elution buffer (1% SDS and 0.1 M $NaHCO_3$) at RT for 15min. The cross-linking was reversed by adding 200mM NaCl and incubated at 56˚C overnight then subsequently digested with 4ul RNase A (100 mg/ml)(Qiagen, Germantown, MD) and 2ul of proteinase K (10mg/ml) at 45˚C for 1hr. After reversing the cross-linking, DNA was isolated using the QIAquick PCR Purification Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. The input and bound DNA fractions were subjected to PCR and analyzed by with specified primer pairs. The following primer set was used to assess HNF-4α binding and H3/H4 lysine acetylation in the *CPT1B* promoter region: 5' primer 5-GGAACCTGACACCTACTCCC-3' and the 3' primer 5- ACATCGGTGACCTTTTCCCT-3'. PPARα and PPARδ binding to the *CPT1B* promoter region was assessed using the EpiTect ChIP qPCR primer assay GPH1022780(-)01A (Qiagen, Germantown, MD).

Statistical analysis

Statistical analyses were performed using PASW Statistics 19 Software (SPSS Inc., Chicago, IL, USA) on raw or log-transformed data. Comparisons between HSkMC from lean and obese donors were performed using independent samples t-tests. All data met assumptions of sphericity and homogeneity of variance. Data are presented as the mean ± SEM.

RESULTS

Participant Characteristics

Participant characteristics are presented in Table 3.1. By design, the obese subjects were heavier and most had a body mass index (BMI) classified as Class III (severe) obesity (≥40 kg/m^2) ($P<0.05$). Fasting blood glucose, cholesterol, and triglyceride values did not differ between groups. However fasting HDL values were significantly lower, while insulin and homeostatic model assessment values were significantly higher in the obese subjects ($P<0.05$). Both groups consisted of relatively young individuals, and were age- and race-matched.

Lipid Oversupply Increases *CPT1B* Gene Expression

In response to the 48h 1:1 oleate:palmitate lipid treatment, there was a significant ($P<0.001$) increase in *CPT1B* mRNA content in both groups (Fig. 3.1); however, in the lipid-treated condition *CPT1B* mRNA content was significantly lower ($P<0.05$) in the obese individuals compared to the lean (Fig. 3.1). Under the control condition (i.e. pre-lipid exposure), there were no differences between the groups in *CPT1B* mRNA.

Lipid Oversupply Remodels DNA Methylation

DNA methylation was determined among 18 cytosines within the *CPT1B* gene (Fig. 3.2A), 16 of which are located within 1000 base pairs (bp) of the transcription start site (TSS). There were no significant differences in basal methylation between the lean and obese individuals. However, in response to the lipid oversupply, there were significant ($P<0.05$) lipid-treatment effects for cytosines at positions 3, 4, 7, 8, 12, 13, and 14 (-299, -295, -152, -134, +211, +223, and +229bp relative to the TSS) where methylation was lower in the lipid-treated

state relative to control. Among the lean individuals (Fig. 3.2B), lipid oversupply resulted in a significant decrease in the methylation of six cytosines at positions 3, 4, 7, 8, 9, and 13 (-299, -295, -152, -134, -83, and +223bp relative to the TSS) and a decrease in methylation of the cytosine at position 14 (+229bp relative to the TSS) which approached significance (P=0.10). In addition, there was a significant increase in methylation at position 2 (-565bp from TSS) and a trend for an increase in methylation at cytosines 17 and 18 (+1,496 and +4321bp from the TSS; P=0.10 and P=0.06) in response to lipid oversupply among the lean women. Among the obese women (Fig. 3.2C), lipid oversupply resulted in a significant decrease in the methylation of cytosines at positions 2, 4, 6, and 14 (-565, -295, -200, and +229bp relative to the TSS) and a trend for a decrease in the cytosine at position 13 (+223bp relative to the TSS; P=0.06). There were no significant increases in methylation at any of the cytosines measured among the obese subjects in response to lipid oversupply.

In terms of *CPT1B* exhibiting differential responses to lipid oversupply with obesity, the change in methylation of the cytosine at position 7 was significantly (P=0.05) different between groups (lean -12.3±4.6% vs. obese -1.5±1.9%) while the change in methylation of the cytosine at position 8 approached significance (lean -8.8±3.5% vs. obese -1.6±1.0%; P=0.07). Additionally, there were significant interactions for cytosines at positions 2 and 6 (-565 and -200bp relative to the TSS) where methylation significantly decreased in the lipid-treated state in HSkMC from the obese, but not the lean subjects. In fact, the lipid-induced change in methylation of the cytosine at position 6 was positively related to the lipid-induced relative change in *CPT1B* gene expression (r=0.71; P<0.01) (Fig. 3.3A). Similarly, the methylation of cytosine 6 in the lipid-treated condition was positively related with the mRNA content of *CPT1B* in the lipid-treated condition (r=0.52; P=0.03) (Fig. 3.3B).

Lipid Oversupply Increases Histone Acetylation and Transcription Factor Binding

In searching for transcriptional regulators of *CPT1B* gene expression in response to lipid oversupply, we focused on promoter associated histone acetylation and transcription factor binding. Overall, in response to the lipid oversupply, there were significant ($P<0.05$) increases in H3/H4 lysine acetylation (Fig. 3.4A) as well as increased HNF-4α (Fig. 3.4B) and PPARδ (Fig. 3.4D) transcription factor binding to the promoter region of *CPT1B* as assessed by chromatin immunoprecipitation (ChIP) assay. There was a significant correlation between the relative change in histone acetylation and the relative changes in PPARδ (Fig. 3.5B) and HNF4-α (Fig. 3.5C) binding to the *CPT1B* promoter region. Lipid oversupply failed to significantly increase PPARα transcription factor binding to the *CPT1B* promoter region (Fig. 3.4C) and there was no correlation between the relative change in histone acetylation and the relative change in PPARα (Fig. 3.5C) binding to the *CPT1B* promoter region. There were no significant differences between lean and obese in terms of *CPT1B* promoter associated histone acetylations and transcription factor binding at baseline or in response to lipid oversupply.

DISCUSSION

In lean individuals, whole-body lipid oxidation increases in response to a high-fat diet, however with obesity, there is an impairment in the ability to adjust to lipid exposure in a similar manner (3, 15). The goal of the present study was to determine if the expression of a rate-limiting enzyme in skeletal muscle mitochondrial FAO, *CPT1B*, differed in a manner indicative of a lack of metabolic flexibility with obesity and if the transcriptional regulation of *CPT1B* could be explained by epigenetic modifications. The main findings of the current study were that: 1) the expression of *CPT1B* was blunted in the severely obese women compared to their lean counterparts in response to lipid oversupply and 2) that changes in CpG methylation, H3/H4 histone acetylation, and transcription factor binding accompanied this response suggesting that *CPT1B* is, at least in part, regulated by epigenetic modifications in human skeletal muscle.

CPT1 exists as three isoforms encoded by separate genes: liver-type (encoded by *CPT1A*), muscle-type (encoded by *CPT1B*), and brain-type (encoded by *CPT1C*). The *CPT1B* gene consists of ~11kb and contains 19 introns and 19 exons, the first of which is non-coding and alternatively transcribed into either: 1) exon 1A (also called U) that is expressed ubiquitously or 2) exon 1B (also called M) that is expressed abundantly in heart and skeletal muscle (16, 17). Long chain fatty acids can regulate *CPT1B* gene expression (16, 18) via PPARα activation and subsequent binding to the peroxisome proliferator response element (PPRE) within the *CPT1B* promoter region (16, 19). Data from Muoio et al. indicates that PPARα and PPARδ play redundant roles in the activation of *CPT1B* gene expression in skeletal muscle (20), while other data suggests that *CPT1B* is preferentially regulated by PPARδ, and not PPARα in skeletal muscle (21). Our findings also suggest that PPARδ may play a more important role in the

transcriptional activation of *CPT1B* compared to PPARα, in response to lipid exposure as we observed a significant increase in PPARδ binding to the *CPT1B* promoter region, but failed to see an increase in PPARα binding (Fig. 3.4).

HNF-4α is a nuclear transcription factor that regulates the expression of several genes involved with energy metabolism and other nuclear receptors, including the *PPAR*s, and *HNF-4α* mutations have been associated with metabolic diseases such as type 2 diabetes and hyperlipidemia (22). The proposed mechanisms by which HNF-4α exerts transcriptional regulation include chromatin structure modulation via histone acetyltransferase recruitment as well as interactions with other transcription factors such as HNF-1, HNF-6, GATA 4, GATA6, p21, PGC-1α and SREBP2 (23). It has been suggested that the transcriptional regulation of some genes involved with lipid metabolism (ie. *ACOT*, *ACOX*, *CD36*, *CPT*, and *ThB*) relies on interactions between the PPARs and HNF-4α (23). For example, Martinez-Jimenez et al. reported that fasting-mediated transcriptional activation of *CPT1* required the synergism of HNF-4α and PPARα (24). While our findings do not definitely show that the lipid-induced transcriptional regulation of *CPT1B* requires the synergism of HNF4α and PPARδ, our data supports a recently described model of *CPT1B* transcriptional regulation proposed by Chamouton and Latruffe (23) where HNF-4α, bound to the PPRE, recruits a ligand-activated PPAR to the promoter in response to altered substrate availability, suggesting that these two receptors act in a crosstalk manner. Additionally, both HNF4-α and PPARδ binding to the *CPT1B* promoter were positively correlated with increased histone acetylation (Fig. 3.5) in response to lipid oversupply which is consistent with the concept that histone acetylation opens the chromatin structure to facilitate accessibility of transcriptional machinery to the promoter regions of genes, thereby regulating activation.

DNA methylation is considered to be a major regulator of transcriptional activity where increased DNA methylation inhibits promoter activity by directly impeding the binding of transcriptional factors to their target sites, consequently reducing gene expression (10). In response to lipid oversupply, there was a significant (P<0.05) increase in *CPT1B* mRNA content (Fig. 3.1), which was accompanied by significant decreases in methylation among seven out of eighteen cytosines measured. Among the obese women only two of the seven cytosines exhibited a lipid-induced decrease in methylation, compared to five out of seven among the lean women (Fig. 3.2). This suggests the overall lipid-induced decrease in methylation was driven by the lean individuals, which is in agreement with *CPT1B* mRNA content being significantly higher in muscle cells derived from lean compared to obese subjects (Fig. 3.1). In addition, the overall extent to which methylation changed in response to lipid oversupply tended to be lower in the obese subjects (Fig. 3.2), which is in accordance with individuals at a greater risk of developing metabolic disease tending to have a lower sensitivity to environmental challenges (ie. high-fat feeding) and acutely regulating DNA methylation (25).

While fatty acids have been shown to increase the transcription of genes that play roles in the oxidation of lipids, glucose can induce the transcriptional response of glycolytic and lipogenic enzymes (26). One transcription factor that has been implicated in the carbohydrate-mediated regulation of glycolytic and lipogeneic genes is upstream stimulatory factor (USF) (27). USFs are members of the basic-helix-loop-helix leucine zipper family of transcription factors, are ubiquitously expressed, and have been implicated in several metabolic diseases (26-29). Using MATCH^{TM} public version 1.0, which is a matrix search for transcription factor binding sites based on TRANSFAC® (30), we identified potential USF binding sites within the *CPT1B* promoter based on sequence analysis and identified two USF binding sites which

contained cytosines at positions 2 and 6. Interestingly, of the cytosines measured in this analysis, these were the only two that showed a significant ($P<0.05$) interaction effect where methylation significantly decreased in the lipid-treated state in HSkMC from the obese, but not the lean subjects. In fact, methylation of the cytosine at position 2 significantly increased among the lean women in response to lipid treatment (Fig. 2), which would suggest a reduction in transcription factor binding at this site. Additionally, the relative change in methylation of the cytosine at position 6 was positively related to the relative change in *CPT1B* mRNA content in response to lipid oversupply (Fig. 3.3). USF has not been previously implicated in the regulation of *CPT1B* in response to lipid treatment. However, data from Putt et al. uncovered a molecular relationship between several metabolic genes and USF in response to nutrient challenges, including a high-fat meal, and suggested that this relationship played a role in the transcriptional fine tuning of these metabolic genes (29). Additionally, it has been suggested that USF may play a role in maintaining the chromatin structure environment at promoter sites and, similar to the PPARs, may switch from activator to repressor depending on which signal transduction pathways are operating (27). While purely speculative at this point, the significant decrease in methylation at two possible USF binding sites among the obese women with lipid supports the notion that USF binding could be acting as a repressor and a partial brake on the lipid-induced upregulation of *CPT1B*. This mechanism may, in part, explain the significantly reduced expression of *CPT1B* mRNA content in response to lipid treatment in skeletal muscle cells derived from obese women (Fig. 3.1).

In summary, by utilizing primary human skeletal muscle cell cultures (HSkMC) we were able to study the transcriptional regulation of *CPT1B* in response to a lipid stimulus in the skeletal muscle of lean and severely obese humans. Our findings indicate a dampened *CPT1B*

response to lipid, which likely contributes to the metabolic *in*flexibility evident in skeletal muscle with severe obesity. A novel finding was that epigenetic modifications, including histone acetylation and DNA methylation, both of which were associated with transcription factor binding, play an important role in the transcriptional upregulation of *CPT1B* in response to a physiologically relevant lipid mixture (31) in humans and it is likely that differential DNA methylation partially explains the depressed expression of *CPT1B* among the severely obese women.

GRANTS

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

DISCLOSURES

The authors have nothing to declare.

REFERENCES

1. Consitt LA, Bell JA, Houmard JA**.** Intramuscular lipid metabolism, insulin action, and obesity. IUBMB Life. 2009;61(1):47-55.

2. Kelley DE, Mandarino LJ**.** Fuel selection in human skeletal muscle in insulin resistance: a reexamination. Diabetes. 2000;49(5):677-83.

3. Battaglia GM, Zheng D, Hickner RC, Houmard JA**.** Effect of exercise training on metabolic flexibility in response to a high-fat diet in obese individuals. Am J Physiol Endocrinol Metab. 2012;303(12):E1440-5. doi: 10.152/ajpendo.00355.2012. Epub 2012 Oct 9.

4. Kim JY, Hickner RC, Cortright RL, Dohm GL, Houmard JA**.** Lipid oxidation is reduced in obese human skeletal muscle. Am J Physiol Endocrinol Metab. 2000;279(5):E1039-44.

5. Consitt LA, Bell JA, Koves TR, Muoio DM, Hulver MW, Haynie KR, et al. Peroxisome proliferator-activated receptor-gamma coactivator-1alpha overexpression increases lipid oxidation in myocytes from extremely obese individuals. Diabetes. 2010;59(6):1407-15. Epub 2010 Mar 3.

6. Zurlo F, Lillioja S, Esposito-Del Puente A, Nyomba BL, Raz I, Saad MF, et al. Low ratio of fat to carbohydrate oxidation as predictor of weight gain: study of 24-h RQ. Am J Physiol. 1990;259(5 Pt 1):E650-7.

7. McGarry JD, Brown NF**.** The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem. 1997;244(1):1-14.

8. Boyle KE, Canham JP, Consitt LA, Zheng D, Koves TR, Gavin TP, et al. A high-fat diet elicits differential responses in genes coordinating oxidative metabolism in skeletal muscle of lean and obese individuals. J Clin Endocrinol Metab. 2011;96(3):775-81. Epub 2010 Dec 29.

9. Vaissiere T, Sawan C, Herceg Z**.** Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat Res. 2008;659(1-2):40-8. Epub 2008 Feb 29.

10. Miranda TB, Jones PA**.** DNA methylation: the nuts and bolts of repression. J Cell Physiol. 2007;213(2):384-90.

11. Evans WJ, Phinney SD, Young VR**.** Suction applied to a muscle biopsy maximizes sample size. Med Sci Sports Exerc. 1982;14(1):101-2.

12. Berggren JR, Tanner CJ, Houmard JA**.** Primary cell cultures in the study of human muscle metabolism. Exerc Sport Sci Rev. 2007;35(2):56-61.

13. Muoio DM, Way JM, Tanner CJ, Winegar DA, Kliewer SA, Houmard JA, et al. Peroxisome proliferator-activated receptor-alpha regulates fatty acid utilization in primary human skeletal muscle cells. Diabetes. 2002;51(4):901-9.

14. Sandoval J, Heyn H, Moran S, Serra-Musach J, Pujana MA, Bibikova M, et al. Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. Epigenetics. 2011;6(6):692-702. Epub 2011 Jun 1.

15. Cameron-Smith D, Burke LM, Angus DJ, Tunstall RJ, Cox GR, Bonen A, et al. A short-term, high-fat diet up-regulates lipid metabolism and gene expression in human skeletal muscle. Am J Clin Nutr. 2003;77(2):313-8.

16. Yu GS, Lu YC, Gulick T**.** Co-regulation of tissue-specific alternative human carnitine palmitoyltransferase Ibeta gene promoters by fatty acid enzyme substrate. J Biol Chem. 1998;273(49):32901-9.

17. van der Leij FR, Cox KB, Jackson VN, Huijkman NC, Bartelds B, Kuipers JR, et al. Structural and functional genomics of the CPT1B gene for muscle-type carnitine palmitoyltransferase I in mammals. J Biol Chem. 2002;277(30):26994-7005. Epub 2002 May 15.

18. Brandt JM, Djouadi F, Kelly DP**.** Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha. J Biol Chem. 1998;273(37):23786-92.

19. Gilde AJ, van der Lee KA, Willemsen PH, Chinetti G, van der Leij FR, van der Vusse GJ, et al. Peroxisome proliferator-activated receptor (PPAR) alpha and PPARbeta/delta, but not PPARgamma, modulate the expression of genes involved in cardiac lipid metabolism. Circ Res. 2003;92(5):518-24. Epub 2003 Feb 6.

20. Muoio DM, MacLean PS, Lang DB, Li S, Houmard JA, Way JM, et al. Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) alpha knock-out mice. Evidence for compensatory regulation by PPAR delta. J Biol Chem. 2002;277(29):26089-97. Epub 2002 May 6.

21. Dressel U, Allen TL, Pippal JB, Rohde PR, Lau P, Muscat GE**.** The peroxisome proliferator-activated receptor beta/delta agonist, GW501516, regulates the expression of genes involved in lipid catabolism and energy uncoupling in skeletal muscle cells. Mol Endocrinol. 2003;17(12):2477-93. Epub 003 Oct 2.

22. Dhe-Paganon S, Duda K, Iwamoto M, Chi YI, Shoelson SE**.** Crystal structure of the HNF4 alpha ligand binding domain in complex with endogenous fatty acid ligand. J Biol Chem. 2002;277(41):37973-6. Epub 2002 Aug 21.

23. Chamouton J, Latruffe N**.** PPARalpha/HNF4alpha interplay on diversified responsive elements. Relevance in the regulation of liver peroxisomal fatty acid catabolism. Curr Drug Metab. 2012;13(10):1436-53.

24. Martinez-Jimenez CP, Kyrmizi I, Cardot P, Gonzalez FJ, Talianidis I**.** Hepatocyte nuclear factor 4alpha coordinates a transcription factor network regulating hepatic fatty acid metabolism. Mol Cell Biol. 2010;30(3):565-77. doi: 10.1128/MCB.00927-09. Epub 2009 Nov 23.

25. Brons C, Jacobsen S, Nilsson E, Ronn T, Jensen CB, Storgaard H, et al. Deoxyribonucleic acid methylation and gene expression of PPARGC1A in human muscle is influenced by high-fat overfeeding in a birth-weight-dependent manner. J Clin Endocrinol Metab. 2010;95(6):3048-56. doi: 10.1210/jc.2009-413. Epub 10 Apr 21.

26. Girard J, Ferre P, Foufelle F**.** Mechanisms by which carbohydrates regulate expression of genes for glycolytic and lipogenic enzymes. Annu Rev Nutr. 1997;17:325-52.

27. Corre S, Galibert MD**.** Upstream stimulating factors: highly versatile stress-responsive transcription factors. Pigment Cell Res. 2005;18(5):337-48.

28. Auer S, Hahne P, Soyal SM, Felder T, Miller K, Paulmichl M, et al. Potential role of upstream stimulatory factor 1 gene variant in familial combined hyperlipidemia and related disorders. Arterioscler Thromb Vasc Biol. 2012;32(6):1535-44. doi: 10.161/ATVBAHA.112.245639. Epub 2012 Mar 29.

29. Putt W, Palmen J, Nicaud V, Tregouet DA, Tahri-Daizadeh N, Flavell DM, et al. Variation in USF1 shows haplotype effects, gene : gene and gene : environment associations with glucose and lipid parameters in the European Atherosclerosis Research Study II. Hum Mol Genet. 2004;13(15):1587-97. Epub 2004 Jun 2.

30. Wingender E**.** The TRANSFAC project as an example of framework technology that supports the analysis of genomic regulation. Brief Bioinform. 2008;9(4):326-32. doi: 10.1093/bib/bbn016. Epub 2008 Apr 24.

31. Watt MJ, Hoy AJ, Muoio DM, Coleman RA**.** Distinct roles of specific fatty acids in cellular processes: implications for interpreting and reporting experiments. Am J Physiol Endocrinol Metab. 2012;302(1):E1-3. doi: 10.1152/ajpendo.00418.2011.

TABLES AND FIGURES

Table 3.1 Subject Characteristics

	Lean (n=12)	**Obese (n=10)**
Age (y)	23.4±1.5	29.9±2.9
Stature (cm)	164.7±1.8	165.5±2.2
Mass (kg)	62.6±1.3	113.7±6.3*
BMI (kg/m^2)	22.8±0.7	41.3±1.5*
Fasting glucose (mmol/L)	4.6±0.1	4.9±0.4
Fasting insulin (uU/L)	4.3±1.2	18.3±5.8*
HOMA-IR	0.9±0.3	3.2±1.0*
Plasma cholesterol (mg/dl)	179±8	169±11
Plasma triglycerides (mg/dl)	102±7	105±16
HDL (mg/dl)	56±4	40±4*

Results are expressed as mean±SEM

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

Fig. 3.1 Effect of lipid oversupply (48hr 250µM oleate:palmitate treatment) on mRNA content of *CPT1B* in cultured myotubes (HSkMC) from lean (open bars) and severely obese (solid bars) donors. Data are expressed as mean±SEM. *, Significant difference ($P \leq 0.05$) between lean and obese individuals. Φ, Significant difference ($P \leq 0.01$) between control and lipid-treated.

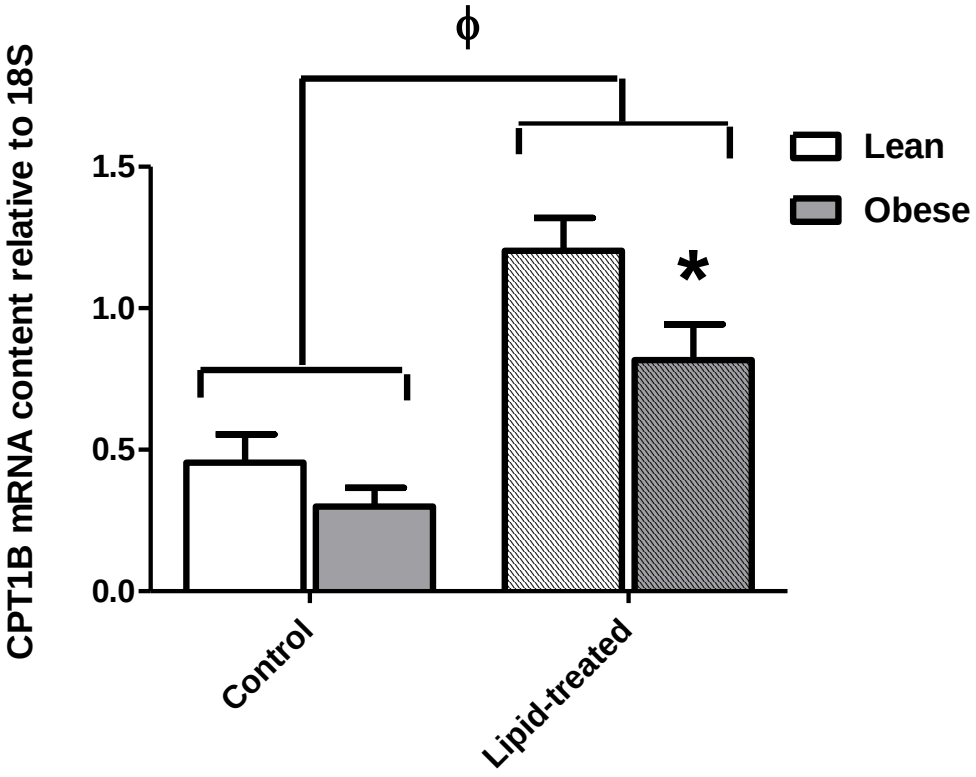
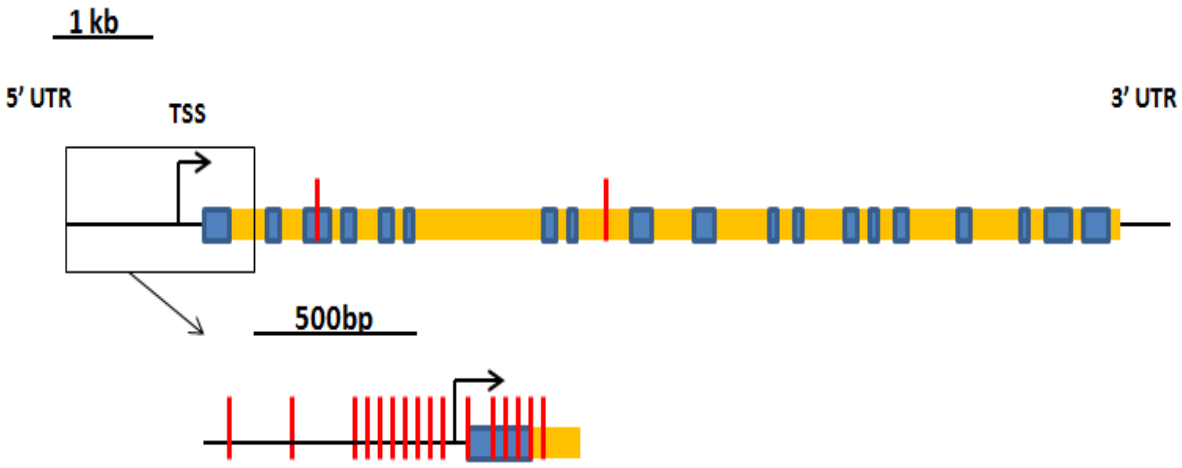
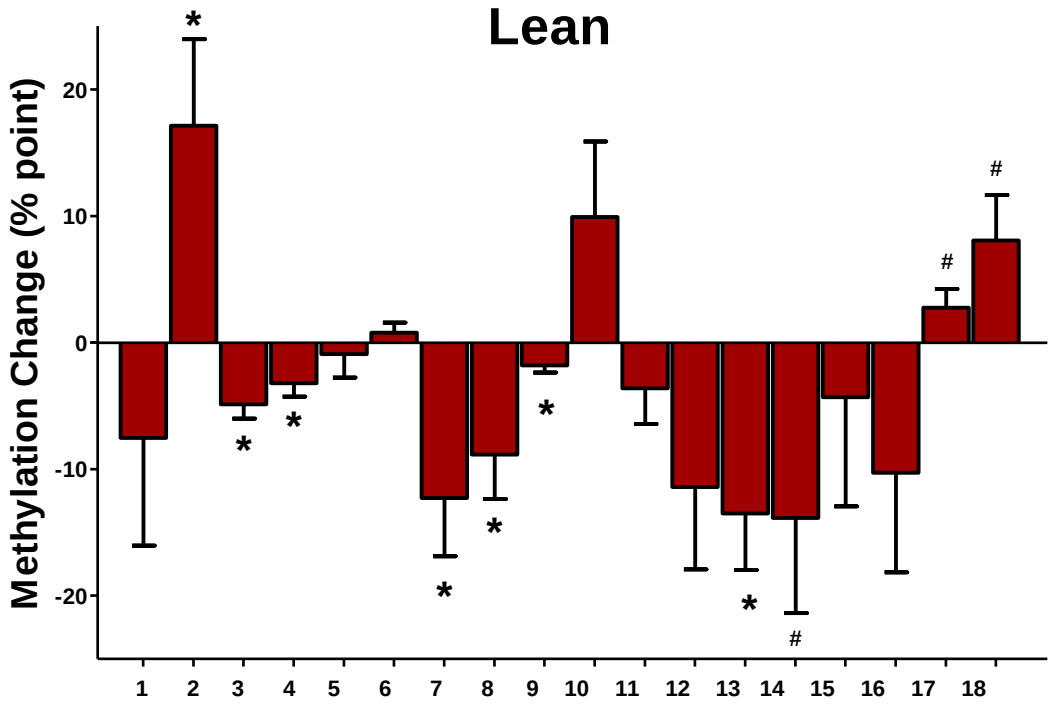


Fig. 3.2 *CPT1B* gene methylation. Visualization of the 18 measured CpG sites within the *CPT1B* gene (A). DNA methylation changes in response to lipid oversupply (methylation value in the lipid-treated condition minus the baseline methylation value) at each of the 18 measured cytosines among the lean individuals (B) and the severely obese individuals (C). *, Significant difference ($P \leq 0.05$) between the baseline and lipid-treated condition. #, Difference between baseline and lipid-treated condition approached significance ($P \leq 0.1$). TSS is indicated by the arrow.

A



B


Lean
Methylation Change (% point)
20
10
0
-10
-20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
*
#

C

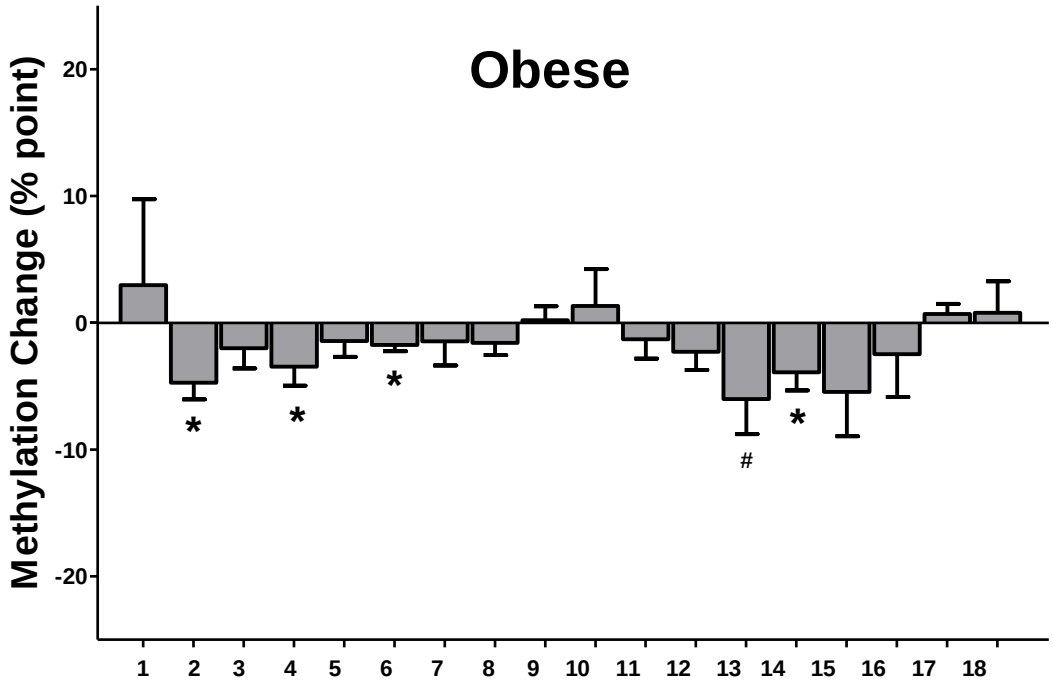
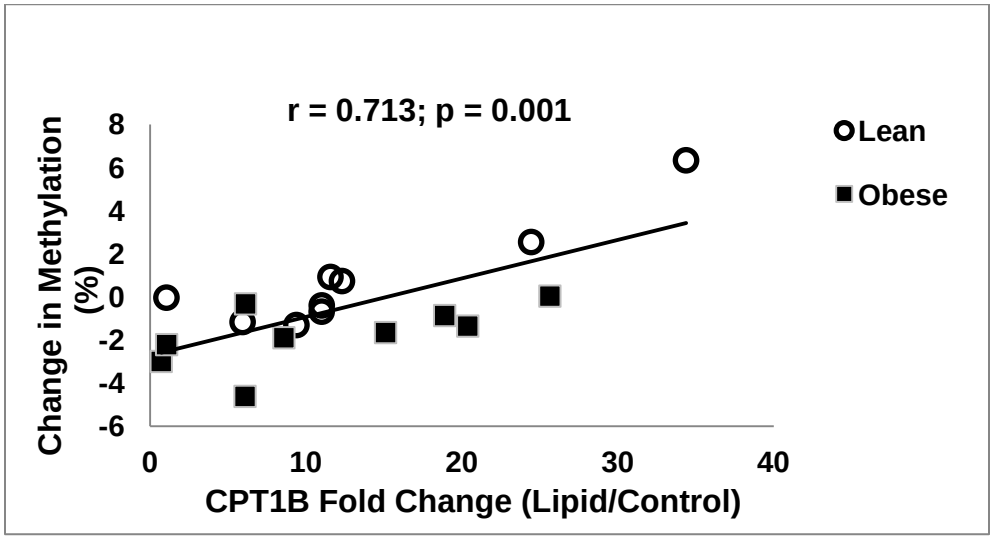

Obese
Methylation Change (% point)
20
10
0
-10
-20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
*
#

Fig. 3.3 The association of *CPT1B* methylation and *CPT1B* mRNA content. The change in methylation in response to lipid oversupply (methylation value in the lipid-treated condition minus the baseline methylation value) of the cytosine at position 6 was positively related to the relative change in *CPT1B* mRNA content (lipid-treated divided by baseline) (A). Methylation of the cytosine at position 6 in the lipid-treated condition was positively related to *CPT1B* mRNA content in the lipid-treated condition (B).

A



B

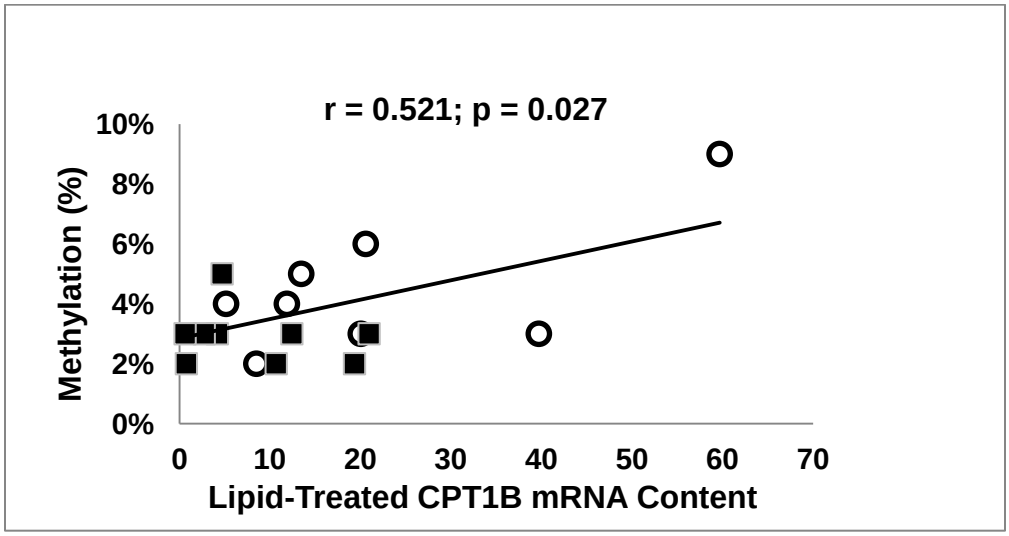


Fig. 3.4 Effect of lipid oversupply (48hr 250μM oleate:palmitate treatment) on *CPT1B* promoter associated changes in H3/H4 histone acetylation (A) and HNF-4α (B), PPARα (C), and PPARδ (D) transcription factor binding in cultured myotubes (HSkMC) from lean (open bars) and severely obese (solid bars) donors. Data are expressed as mean±SEM. Φ, Significant difference ($P \leq 0.05$) between control and lipid-treated.

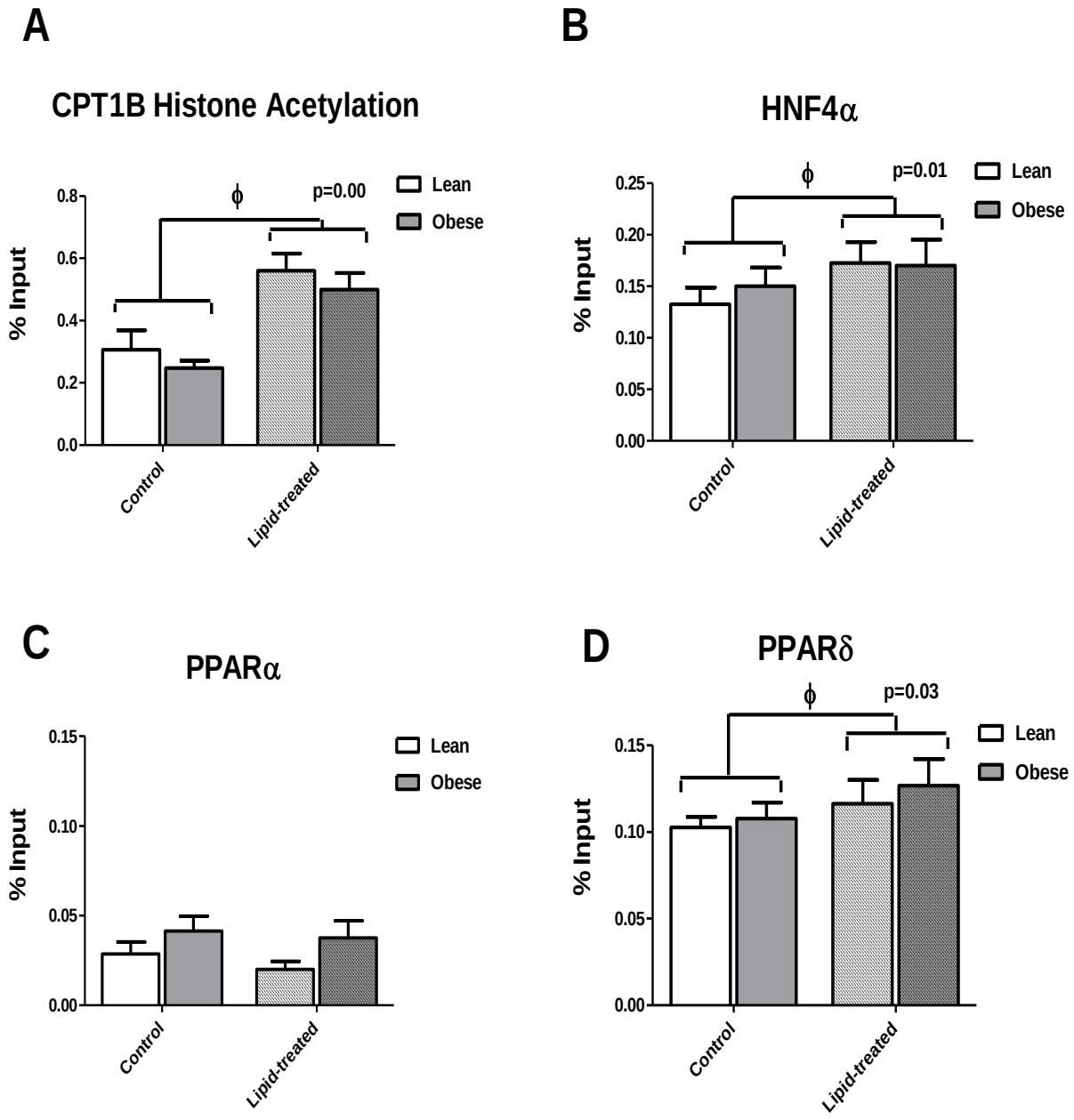
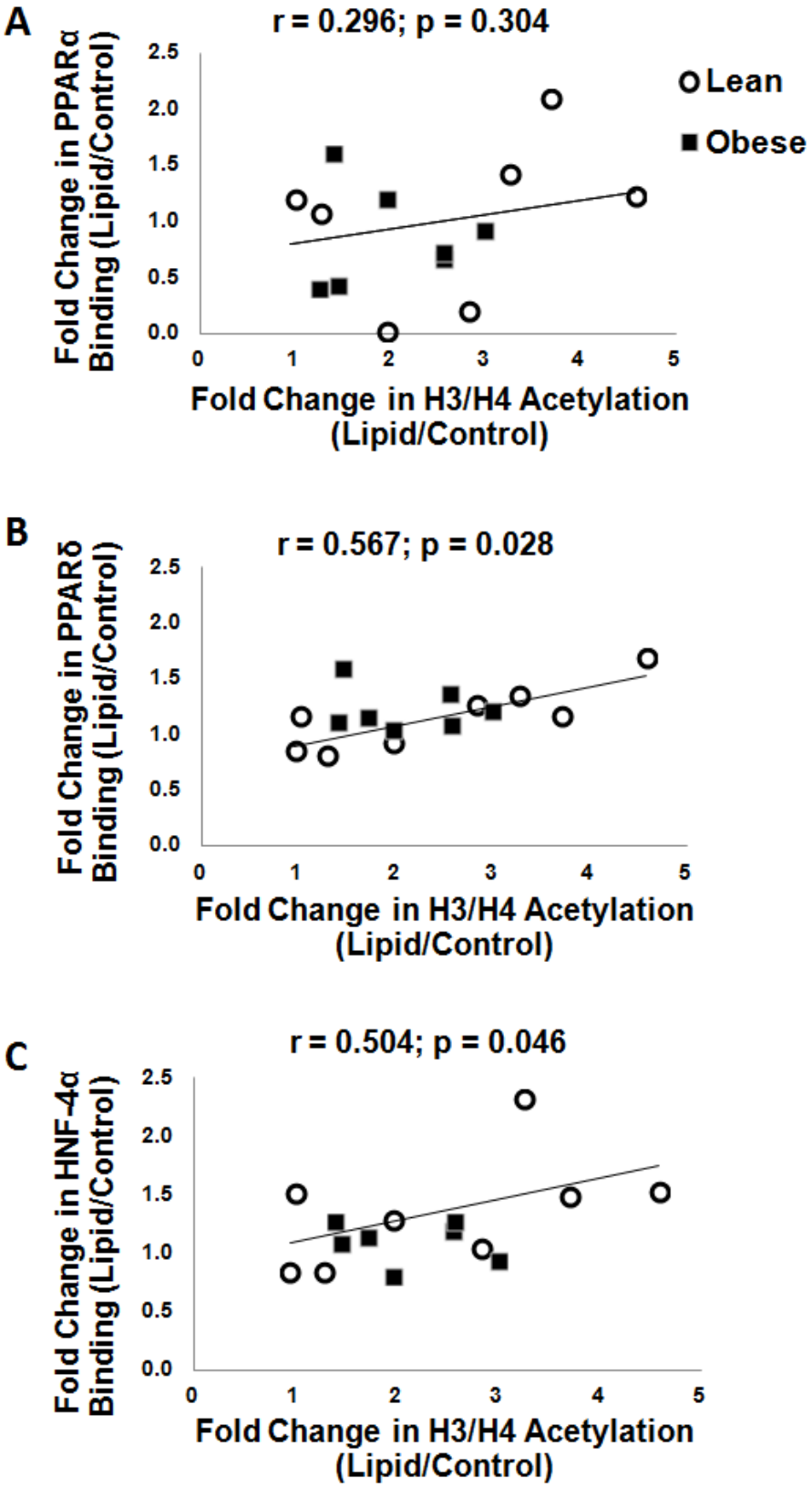


Fig. 3.5 The association of *CPT1B* promoter region H3/H4 histone acetylation and transcription factor binding to the *CPT1B* promoter region. The relative change (lipid-treated divided by baseline) in PPARα (A), PPARδ (B), and HNF-4α (C) transcription factor binding to the *CPT1B* promoter region in response to lipid oversupply was related to the relative change (lipid-treated divided by baseline) in H3/H4 histone acetylation of the *CPT1B* promoter region.


A
r = 0.296; p = 0.304
Lean
Obese
Fold Change in PPARα Binding (Lipid/Control)
Fold Change in H3/H4 Acetylation (Lipid/Control)
B
r = 0.567; p = 0.028
Fold Change in PPARδ Binding (Lipid/Control)
Fold Change in H3/H4 Acetylation (Lipid/Control)
C
r = 0.504; p = 0.046
Fold Change in HNF-4α Binding (Lipid/Control)
Fold Change in H3/H4 Acetylation (Lipid/Control)

CHAPTER 4: SUMMARY OF FINDINGS

With obesity, there are impairments in the ability to adjust substrate utilization to changes in substrate availability, referred to as metabolic *in*flexibility (1-3). In respect to lipid metabolism, we reported that obese individuals lacked the ability to increase fat oxidation with either a high fat diet (4) or in HSkMC upon lipid incubation (5). This dissertation examined if the expression of genes linked with FAO and mitochondrial content also differed in a manner indicative of a lack of metabolic flexibility with severe obesity and to what extent the differential responses to lipid oversupply were linked with the chromatin environment and/or the methylation signature of these genes. The main findings were that: 1) the increased expression of genes that act as vital transcriptional and enzymatic regulators of FAO and mitochondrial content were blunted in the severely obese women compared to their lean counterparts in response to lipid oversupply and 2) that changes in CpG methylation, H3/H4 histone acetylation, and transcription factor binding were evident in this lipid-induced response suggesting that some genes (ie. *CPT1B* and *PPARδ*) are, at least in part, regulated by epigenetic modifications in human skeletal muscle. These data provide the novel information that with severe obesity the metabolic *in*flexibility evident with lipid exposure may be linked with an inability to upregulate some transcriptional regulators via epigenetic modifications.

The first study in this dissertation focused on the peroxisome proliferator-activated receptors (PPARs) and nuclear respiratory factors (NRFs) , which are provocative candidates for explaining the metabolic *in*flexibility in response to lipid oversupply with obesity as they activate gene expression programs critical to mitochondrial function and FAO (6-9). The NRFs are essential for the expression several genes that are critical to FAO including genes that encode subunits of the five respiratory complexes of the mitochondrial inner membrane, genes which

direct the expression and assembly of the respiratory apparatus, and genes which function in mitochondrial protein import and initiate mtDNA transcription and replication (10, 11). *NRF-1,* and *NRF-2*, exhibited patterns of increasing mRNA content with lipid exposure in the lean, but not in HSkMC from the severely obese (Fig. 2.1). Of the six NRF-regulated genes examined, cytochrome c (*CYCS*) exhibited a similar pattern of increasing mRNA content with lipid oversupply in the lean, but decreasing with obesity (Fig. 2.3). We cannot discount the possibility that these findings may have been influenced by the time we chose to obtain the samples, i.e. that similar or more divergent mRNA responses in lean and obese subjects could have occurred at earlier or later time points during lipid incubation.

The PPARs are ligand-activated transcription factors that play essential roles in lipid homeostasis by modulating the expression of genes that regulate fatty acid catabolism. There are three PPAR subtypes: 1) PPARα, which mediates lipid-induced activation of FAO genes and is expressed predominately in tissues that are characterized by high rates of FAO (ie. liver, heart, muscle, kidney); 2) PPARγ, which is highly enriched in adipocytes and macrophages and is involved in adipocyte differentiation, lipid storage, and glucose homeostasis; and 3) PPARδ, which is ubiquitously expressed and has the least defined function, but has recently been characterized as being highly expressed in skeletal muscle and playing a lipid-metabolizing role similar to PPARα (7, 8, 12). Their critical role in energy homeostasis is supported by the observation that *PPARα* knockout mice exhibit a dramatic inhibition of fatty acid uptake and oxidation, abnormal accumulation of lipids in oxidative tissues, and a failure to induce beta-oxidation in response to physiological challenges such as a high-fat diet (13). In the first study, the mRNA content of *PPARα* and *PPARδ* increased with lipid exposure in the lean, but decreased in HSkMC from the severely obese (Fig. 2.1 and 2.2). Additionally, several PPAR-

responsive genes including citrate synthase (*CS),* mitochondrial uncoupling protein 3 (*UCP3),* and pyruvate dehydrogenase kinase 4 (*PDK4)* showed trends for being upregulated more robustly in HSkMC from lean but not obese subjects (Fig. 2.3).

Of the PPAR-responsive genes that were differentially regulated by the 48hr lipid oversupply *PDK4* is of particular interest as it suppresses glucose and promotes fat oxidation in the presence of lipids (14). We have previously shown that a high fat diet increased *PDK4* mRNA content in lean, but not obese individuals (15); in the current study utilizing HSkMC, the lipid-induced increase in *PDK4* mRNA content in the lean was more than 2-fold greater than the response in the obese (42.0±8.1 vs. 20.4±3.8 fold increase) (Fig. 2.3). This data is consistent with an inability to switch from predominately glucose oxidation to FAO in the presence of a lipid challenge with obesity.

In the second study of this dissertation, the response to another PPAR-responsive gene, the muscle-type carnitine palmitoyltransferase 1B (*CPT1B*), was evaluated. CPT1 mediates the transfer of long chain fatty acids across the outer mitochondrial membrane which is a rate-limiting step in FAO (16). The activity of the muscle-type CPT1 (encoded by the *CPT1B* gene) is reduced in skeletal muscle with obesity, contributing to a decrease in FAO (17). In relation to metabolic flexibility, in the skeletal muscle of lean subjects a high fat diet increased the expression of *CTP1B* and the peroxisome proliferator-activated receptors (*PPAR*s) which are upstream regulators of *CPT1B* expression (15). Conversely, a high fat diet resulted in little to no change in the expression of these genes in the skeletal muscle of severely obese subjects (15). Our data showed that in response to lipid treatment, there was a significant increase in *CPT1B* mRNA content in both groups (Fig. 3.1); however, in the lipid-treated condition *CPT1B* mRNA

content was significantly lower in the obese individuals compared to the lean (Fig. 3.1). Our findings indicate a dampened *CPT1B* response to lipid which likely contributes to the metabolic *in*flexibility evident in skeletal muscle with severe obesity.

Taken together, our findings indicate a coordinated activation of genes linked with FAO and mitochondrial content among lean individuals in response to lipid oversupply that is largely absent with obesity. In addition to our examination of the differential lipid-induced upregulation of genes linked with FAO and mitochondrial content among lean and obese women, we examined to what extent the differential responses to lipid oversupply were linked with the chromatin environment and/or the methylation signature of these genes. Epigenetic modifications of the genome, including DNA methylation and histone acetylation, may provide a connection between nutritional factors, gene expression, and metabolic health.

DNA methylation is considered to be a major regulator of transcriptional activity and may provide a mechanism for the regulation of gene expression in response to lipid and/or could help explain the differential responses in gene expression in the skeletal muscle of lean vs. severely obese individuals. Our data, along with others (18, 19), supports the growing awareness that DNA methylation can be viewed as a dynamic signal that is associated with changes in substrate availability, such as lipid oversupply. However, the relationship of gene expression with DNA methylation, as well as the precise functional relevance of differential methylation is not straightforward. For example, Jacobsen et al. (19) observed that, in response to a high-fat diet, relatively immediate changes in DNA methylation can be slow to reverse, but the functional relevance of this slow reversibility is not known (19). The authors suggest that perhaps the methylation changes induced by the high-fat diet actually prevented potentially detrimental

effects of the high-fat diet on gene expression (19). In our study, the overall extent to which methylation changed in response to lipid oversupply tended to be lower in the obese subjects in both studies (Fig. 2.4 and 3.2). This finding is in accordance with previous work showing that individuals at a greater risk of developing metabolic disease tended to have a lower sensitivity to environmental challenges (ie. high-fat feeding) in terms of the ability to regulate changes in DNA methylation (20). We did not assess the reversibility of changes in methylation, but future studies addressing the functional relevance of lingering changes in methylation could give us additional insight into the lipid-induced regulation of the genes examined in this dissertation.

DNA methylation is generally accepted to regulate gene transcription by directly impeding the binding of transcriptional factors to their target sites and through the recruitment of methyl-binding proteins (21). However, our findings indicated a positive relationship between the extent of *PPARδ* promoter methylation and *PPARδ* mRNA content in response to lipid oversupply (Fig. 2.6). Additionally, the extent of methylation of one cytosine -200bp relative to the TSS in the *CPT1B* promoter region was positively related with the mRNA content of *CPT1B* in the lipid-treated condition (Fig. 3.3). While purely speculative at this point, the divergent methylation signatures between the lean and obese women of cytosines in the promoter regions of *CPT1B* and *PPARδ*, that are positively correlated with mRNA content, supports the notion that methylation may be impeding the binding of an unknown repressor. Future studies aimed at identifying these potential repressors could shed light on the regulation of these genes, which play vital roles in lipid metabolism, and could provide additional insight into mechanisms responsible for the metabolic *in*flexibility evident with severe obesity. With this in mind, it is intriguing to imagine if, in the future, therapeutic modalities to treat obesity and/or metabolic *in*flexibility might include approaches to manipulate the methylation status of a single nucleotide

within the promoter region of a targeted gene, in an effort to modify promoter occupancy, in turn regulating the transcriptional activity of the gene.

Another epigenetic modification, histone acetylation, also is considered to be a major regulator of transcriptional activity via modulation of chromatin structure which influences the interaction of transcription-regulatory proteins with target DNA in chromatin (22). For example, an increase in the acetyl groups on histones will result in an open chromatin structure to facilitate accessibility of transcriptional machinery to DNA templates in chromatin, in turn increasing the expression of a gene. Overall, in response to the lipid oversupply, there were significant increases among the lean and obese groups in H3/H4 lysine acetylation (Fig. 3.4) as well as increased HNF-4α (Fig. 3.4) and PPARδ (Fig. 3.4) transcription factor binding to the promoter region of *CPT1B*. Additionally, both HNF4-α and PPARδ binding to the *CPT1B* promoter were positively correlated with histone acetylation (Fig. 3.5) in response to lipid oversupply which is consistent with the concept that increasing histone acetylation opens the chromatin structure to facilitate accessibility of transcriptional machinery to the promoter regions of genes, thereby regulating activation.

HNF-4α is a nuclear transcription factor that regulates the expression of several genes involved with energy metabolism and other nuclear receptors, including the PPARs, and *HNF-4α* mutations have been associated with metabolic diseases such as Type II diabetes and hyperlipidemia (23). The proposed mechanisms by which HNF-4α exerts transcriptional regulation include chromatin structure modulation via histone acetyltransferase recruitment as well as interactions with other transcription factors such as the PPARs (24). For example, Martinez-Jimenez et al. reported that fasting-mediated transcriptional activation of *CPT1*

required the synergism of HNF-4α and PPARα (25). Our findings did not definitely show that the lipid-induced transcriptional regulation of *CPT1B* requires the synergism of a PPAR and HNF4α, however our data supports a recently described model of *CPT1B* transcriptional regulation (24) where HNF-4α, bound to the Peroxisome Proliferator Response Element (PPRE), recruits a ligand-activated PPAR to the promoter in response to altered substrate availability. Our data suggests that PPARδ, not PPARα, is the ligand-activated PPAR recruited to the *CPT1B* promoter region in response to lipid oversupply, indicating that PPARδ may play a more important role in the lipid-induced transcriptional activation of *CPT1B* compared to PPARα. Together these data indicate that histone acetylation, which is associated with HNF-4α and PPARδ transcription factor binding, plays an important role in the transcriptional upregulation of *CPT1B* in response to a physiologically relevant lipid mixture (26) in humans.

As mentioned previously, PPARδ has the least defined function of the three PPAR subtypes. In light of this, it is particularly interesting that *PPARδ* was the only gene whose mRNA content was significantly lower in the obese individuals compared to the lean at baseline *and* in response to lipid oversupply (Fig. 2.2). There was a significant interaction effect for *PPARδ* where mRNA content was elevated in the lipid-treated state in HSkMC from the lean, but decreased in HSkMC from the obese subjects (Fig. 2.2). Additionally, PPARδ, not PPARα, binding to the *CPT1B* promoter region increased in response to lipid oversupply and was significantly correlated with *CPT1B* mRNA content in response to lipid exposure. Together this data indicates that *PPARδ* may play a more important role in the transcriptional activation of *CPT1B*, and perhaps in skeletal muscle metabolic flexibility and lipid metabolism, than previously thought. Not surprisingly, along with PPARα and PPARγ agonists, PPARδ agonists

are currently under assessment in clinical trials and seem to be promising drugs for the improvement of parameters associated with dyslipidemia, insulin resistance, and obesity (27).

In summary, by utilizing primary human skeletal muscle cell cultures (HSkMC) we were able to study the transcriptional regulation of mitochondrial and lipid-oxidizing genes in response to a lipid stimulus in the skeletal muscle of lean and severely obese humans in an environment void of *in-vivo* hormonal and neural stimuli and thus intrinsic to skeletal muscle itself. The purpose of the present study was to determine if the expression of genes linked with FAO and mitochondrial content differed in a manner indicative of a lack of metabolic flexibility with obesity and to what extent the differential responses to lipid oversupply were linked with the chromatin environment and/or the methylation signature of these genes. The main findings were that: 1) the coordinated activation of genes linked with FAO and mitochondrial content among lean individuals in response to lipid oversupply is largely absent with obesity and 2) that changes in CpG methylation, increased histone acetylation, and transcription factor binding accompanied this response, suggesting that epigenetic modifications play a role in the lipid-induced upregulation of these genes. These data provide the novel information that with severe obesity the metabolic *in*flexibility evident in response to lipid exposure may be linked with an inability to upregulate transcriptional regulators caused by differential epigenetic modifications.

REFERENCES

1. Astrup A, Buemann B, Christensen NJ, Toubro S**.** Failure to increase lipid oxidation in response to increasing dietary fat content in formerly obese women. Am J Physiol. 1994;266(4 Pt 1):E592-9.

2. Kelley DE**.** Skeletal muscle fat oxidation: timing and flexibility are everything. J Clin Invest. 2005;115(7):1699-702.

3. Ravussin E**.** Metabolic differences and the development of obesity. Metabolism. 1995;44(9 Suppl 3):12-4.

4. Battaglia GM, Zheng D, Hickner RC, Houmard JA**.** Effect of exercise training on metabolic flexibility in response to a high-fat diet in obese individuals. Am J Physiol Endocrinol Metab. 2012;303(12):E1440-5. doi: 10.152/ajpendo.00355.2012. Epub 2012 Oct 9.

5. Boyle KE, Zheng D, Anderson EJ, Neufer PD, Houmard JA**.** Mitochondrial lipid oxidation is impaired in cultured myotubes from obese humans. Int J Obes. 2011;25(10):201.

6. Kelly DP, Scarpulla RC**.** Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev. 2004;18(4):357-68.

7. Muoio DM, MacLean PS, Lang DB, Li S, Houmard JA, Way JM, et al. Fatty acid homeostasis and induction of lipid regulatory genes in skeletal muscles of peroxisome proliferator-activated receptor (PPAR) alpha knock-out mice. Evidence for compensatory regulation by PPAR delta. J Biol Chem. 2002;277(29):26089-97. Epub 2002 May 6.

8. Lee CH, Olson P, Evans RM**.** Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. Endocrinology. 2003;144(6):2201-7.

9. Muoio DM, Koves TR**.** Skeletal muscle adaptation to fatty acid depends on coordinated actions of the PPARs and PGC1 alpha: implications for metabolic disease. Appl Physiol Nutr Metab. 2007;32(5):874-83.

10. Scarpulla RC**.** Transcriptional paradigms in mammalian mitochondrial biogenesis and function. Physiol Rev. 2008;88(2):611-38.

11. Scarpulla RC**.** Nuclear control of respiratory gene expression in mammalian cells. J Cell Biochem. 2006;97(4):673-83.

12. Wagner KD, Wagner N**.** Peroxisome proliferator-activated receptor beta/delta (PPARbeta/delta) acts as regulator of metabolism linked to multiple cellular functions. Pharmacol Ther. 2010;125(3):423-35. doi: 10.1016/j.pharmthera.2009.12.001. Epub Dec 22.

13. Muoio DM, Way JM, Tanner CJ, Winegar DA, Kliewer SA, Houmard JA, et al. Peroxisome proliferator-activated receptor-alpha regulates fatty acid utilization in primary human skeletal muscle cells. Diabetes. 2002;51(4):901-9.

14. Sugden MC, Holness MJ**.** Mechanisms underlying regulation of the expression and activities of the mammalian pyruvate dehydrogenase kinases. Arch Physiol Biochem. 2006;112(3):139-49.

15. Boyle KE, Canham JP, Consitt LA, Zheng D, Koves TR, Gavin TP, et al. A high-fat diet elicits differential responses in genes coordinating oxidative metabolism in skeletal muscle of lean and obese individuals. J Clin Endocrinol Metab. 2011;96(3):775-81. Epub 2010 Dec 29.

16. McGarry JD, Brown NF**.** The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem. 1997;244(1):1-14.

17. Kim JY, Hickner RC, Cortright RL, Dohm GL, Houmard JA**.** Lipid oxidation is reduced in obese human skeletal muscle. Am J Physiol Endocrinol Metab. 2000;279(5):E1039-44.

18. Barres R, Osler ME, Yan J, Rune A, Fritz T, Caidahl K, et al. Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density. Cell Metab. 2009;10(3):189-98.

19. Jacobsen SC, Brons C, Bork-Jensen J, Ribel-Madsen R, Yang B, Lara E, et al. Effects of short-term high-fat overfeeding on genome-wide DNA methylation in the skeletal muscle of healthy young men. Diabetologia. 2012;55(12):3341-9. doi: 10.1007/s00125-012-2717-8. Epub 2012 Sep 8.

20. Brons C, Jacobsen S, Nilsson E, Ronn T, Jensen CB, Storgaard H, et al. Deoxyribonucleic acid methylation and gene expression of PPARGC1A in human muscle is influenced by high-fat overfeeding in a birth-weight-dependent manner. J Clin Endocrinol Metab. 2010;95(6):3048-56. doi: 10.1210/jc.2009-413. Epub 10 Apr 21.

21. Miranda TB, Jones PA**.** DNA methylation: the nuts and bolts of repression. J Cell Physiol. 2007;213(2):384-90.

22. Vaissiere T, Sawan C, Herceg Z**.** Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat Res. 2008;659(1-2):40-8. Epub 2008 Feb 29.

23. Dhe-Paganon S, Duda K, Iwamoto M, Chi YI, Shoelson SE**.** Crystal structure of the HNF4 alpha ligand binding domain in complex with endogenous fatty acid ligand. J Biol Chem. 2002;277(41):37973-6. Epub 2002 Aug 21.

24. Chamouton J, Latruffe N**.** PPARalpha/HNF4alpha interplay on diversified responsive elements. Relevance in the regulation of liver peroxisomal fatty acid catabolism. Curr Drug Metab. 2012;13(10):1436-53.

25. Martinez-Jimenez CP, Kyrmizi I, Cardot P, Gonzalez FJ, Talianidis I**.** Hepatocyte nuclear factor 4alpha coordinates a transcription factor network regulating hepatic fatty acid metabolism. Mol Cell Biol. 2010;30(3):565-77. doi: 10.1128/MCB.00927-09. Epub 2009 Nov 23.

26. Watt MJ, Hoy AJ, Muoio DM, Coleman RA**.** Distinct roles of specific fatty acids in cellular processes: implications for interpreting and reporting experiments. Am J Physiol Endocrinol Metab. 2012;302(1):E1-3. doi: 10.1152/ajpendo.00418.2011.

27. Gross B, Staels B**.** PPAR agonists: multimodal drugs for the treatment of type-2 diabetes. Best Pract Res Clin Endocrinol Metab. 2007;21(4):687-710.

APPENDIX: IRB APPROVAL

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

Notification of Continuing Review Approval

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

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

The Biomedical IRB deemed this study Greater than Minimal Risk.

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

The approval includes the following items:

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

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

EAST CAROLINA UNIVERSITY
University & Medical Center Institutional Review Board Office
4N-70 Brody Medical Sciences Building · Mail Stop 682
600 Moye Boulevard · Greenville, NC 27834
Office **252-744-2914** · Fax **252-744-2284** · **www.ecu.edu/irb**

Notification of Continuing Review Approval

From: Biomedical IRB
To: Joseph Houmard
CC:

Gabriel Dubis
Date: 12/13/2012
Re: CR00000684
UMCIRB 06-0080
Lipid Metabolism in Obesity, Weight Loss and Exercise (2): Muscle Cell Studies

I am pleased to inform you that at the convened meeting on 12/12/2012 of the Biomedical IRB, this research study underwent a continuing review and the committee voted to approve the study. Approval of the study and the consent form(s) is for the period of 12/12/2012 to 12/11/2013.

The Biomedical IRB deemed this study Greater than Minimal Risk.

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

The approval includes the following items:

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

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

R. Hickner

The following UMCIRB members with a potential Conflict of Interest did not attend this IRB meeting:

None