Catabolic and Compensatory Metabolic Events in Mice during Conditions of Cachexia and Food Restriction

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Kara Lea Kliewer

The Ohio State University Nutrition Program

The Ohio State University

2014

Dissertation Committee:

Dr. Martha A. Belury, Advisor

Dr. Denis Guttridge

Dr. Earl Harrison

Dr. Kichoon Lee
Copyright by
Kara Lea Kliewer
2014
Abstract

Cancer cachexia is a metabolic disorder characterized by extensive depletion of adipose tissue and skeletal muscle mass that is associated with significant morbidity and mortality. A growing body of literature suggests that maintaining fat mass in cachexia may improve quality-of-life and survival outcomes, but the metabolic factors and biological mechanisms underlying the loss of adipose tissue in cachexia have been minimally investigated. The goal of this research was to better understand the factors promoting lipolysis (catabolism of triglyceride stores) and lipid utilization in adipose tissues in the early stage of cancer-induced cachexia – processes that may contribute to delipidation of adipose tissue and thus reduce survival.

The first objective of this research was to characterize lipolysis in white adipose tissue in a murine model of early cancer cachexia. Male CD2F1 mice were inoculated with $1 \times 10^6$ colon-26 carcinoma cells and sacrificed at the onset of anorexia and weight loss. In this study of early cachexia, adipose tissue mass in cachectic mice was moderately reduced. Total energy expenditure was elevated compared to pair-fed controls despite reduced locomotor activity. Basal (unstimulated) but not isoproterenol stimulated lipolysis was increased in adipose tissue explants of cachectic mice compared to ad-libitum fed controls, but rates were not different than in pair-fed controls. Gene and protein
expression of lipases were unchanged. These data suggest that lipolysis in early cachexia is the result of an energy deficit from hypermetabolism and reduced food intake rather than changes in expression of key enzymes promoting lipolysis.

The second objective was to characterize processes associated with lipid utilization within adipose tissues in cachexia. In the same study used for the first objective, markers of fatty acid uptake, mitochondrial biogenesis, fatty acid oxidation, respiration, and adaptive thermogenesis were increased in brown adipose tissue of tumor-bearing mice compared to pair-fed controls. Uncoupling protein 1 protein expression in tumor-bearing mice correlated with basal lipolysis rate. Whole-body lipid utilization was increased and markers of fatty acid oxidation in white adipose tissue were elevated. Expression of markers of mitochondrial biogenesis and adaptive thermogenesis, however, were unchanged or decreased in white adipose tissue of cachectic mice. These data suggest that compensatory mechanisms in response to reduced food intake that suppress brown adipose tissue thermogenesis may be lost in tumor-bearing mice and contribute to the depletion of adipose tissue in cachexia.

The third objective was to assess feeding behavior and characterize changes in adiposity in healthy mice subjected to a restricted feeding regimen. Male CD2F1 mice were food restricted for 5 days and fed controlled rations for an additional 12 days. Food restriction triggered gorging behavior. Despite reduced cumulative food intake compared to ad-libitum fed mice, intra-abdominal fat mass was increased in restricted mice which was
accompanied by elevated lipogenic and reduced uncoupling protein 1 gene expression. Hepatic and peripheral insulin sensitivity were diminished and insulin levels trended higher. These data suggest enhanced fat accumulation in food restricted mice may be explained by a compensatory suppression of thermogenesis (thus energy expenditure) and a gene expression profile favoring lipid deposition.
Acknowledgments

I would like to thank my advisor, Dr. Martha Belury, for providing me the opportunity to conduct this research, numerous opportunities to learn new skills and collaborate with other researchers, and for reviewing countless applications, abstracts, posters, and papers. I would also like to sincerely thank Dr. Belury and members of my committee, Dr. Denis Guttridge, Dr. Earl Harrison and Dr. Kichoon Lee for their time and advice from start to finish.

I would like to thank my colleagues Michelle and Min for their unwavering support and for laying the groundwork for this research. I would like to especially thank Holly whose support, suggestions, help with experiments, and friendship has been invaluable the last 3 years. I am indebted to Rachel for her expert training on proper lab techniques and help on a daily basis.

Thank you to the organizations that contributed financially to support my education and research: The Graduate School University Fellowship, OARDC Research Associateship Program Award, Pelotonia Graduate Fellowship, Minnie Price and Alpha and Paul E. Haffey Research Scholarships, and the College of Education and Human Ecology.
Finally, I would like to thank my friends (Fabi, Holly, and Elizabeth), my family (Lisa and Haley), and my parents for their encouragement, support, and laughs.
Vita

1987 ........................................... B.A. Mathematics, Washington University, St. Louis, MO

2006 ........................................... B.S. Dietetics, University of Northern, Colorado, Greeley, CO

2006 - 2007 ................................... Dietetic Intern, Barnes Jewish College, St. Louis, MO

2007 - 2009 ................................... Clinical Dietitian, OSF St. Francis Hospital, Peoria, IL

2009 - 2014 ................................... Graduate Research Associate, The Ohio State University, Columbus, OH

2009 - 2010 ................................... Graduate School University Fellowship, The Ohio State University, Columbus, OH

2009 - 2010 ................................... Minnie Price Scholarship, The Ohio State University, Columbus, OH

2009 - 2010 ................................... Alpha and Paul E. Haffey Scholarship, The Ohio State University, Columbus, OH
2010 - 2011 ........................................OARDC Director’s Associateship Award,

The Ohio State University, Columbus, OH

2011 - 2013 ............................................Pelotonia Fellowship Award,

The Ohio State University, Columbus, OH

2013 - 2014 ............................................EHE Dissertation Research Fellowship,

The Ohio State University, Columbus, OH

Publications


Fields of Study

Major Field: The Ohio State University Nutrition Program
Table of Contents

Abstract...................................................................................................................... ii

Acknowledgments...................................................................................................... v

Vita................................................................................................................................. vii

List of Tables ................................................................................................................ xi

List of Figures ............................................................................................................... xii

Chapter 1  Introduction ............................................................................................... 1

1.1 Introduction............................................................................................................. 1

1.2 Aims ......................................................................................................................... 4

Chapter 2  Literature Review ..................................................................................... 6

2.1 Cancer Cachexia .................................................................................................... 6

2.2 Lipolysis ................................................................................................................ 13

2.3 Lipid Utilization ..................................................................................................... 21

2.4 Food Restriction in Mice ...................................................................................... 26

Chapter 3  Increased Metabolic Rate and Lipolysis Associated with the Depletion of
White Adipose Tissue in Early Cancer Cachexia ....................................................... 30
### Abstract

3.1 Abstract ........................................................................................................................................... 31

### Introduction

3.2 Introduction ....................................................................................................................................... 32

### Materials and Methods

3.3 Materials and Methods .................................................................................................................... 34

### Results

3.4 Results ................................................................................................................................................ 40

### Discussion

3.5 Discussion .......................................................................................................................................... 59

### Acknowledgements

3.6 Acknowledgements ............................................................................................................................ 64

---

### Chapter 4

**Short-term Food Restriction with Controlled Refeeding Triggers Gorging Behavior, Enhances Fat Deposition, and Diminishes Insulin Sensitivity in Mice**

#### Abstract

4.1 Abstract .............................................................................................................................................. 66

#### Introduction

4.2 Introduction ....................................................................................................................................... 68

#### Materials and Methods

4.3 Materials and Methods .................................................................................................................... 70

#### Results

4.4 Results ................................................................................................................................................ 76

#### Discussion

4.5 Discussion .......................................................................................................................................... 88

#### Acknowledgements

4.6 Acknowledgements ............................................................................................................................ 94

---

### Chapter 5

**Epilogue**

Chapter 5 Epilogue .................................................................................................................................... 95

### List of References

List of References ....................................................................................................................................... 98

### Appendix A

**Lipolysis Assay** ....................................................................................................................................... 111

### Appendix B

**Lipolysis after 24 h Fast** .................................................................................................................... 115

### Appendix C

**Adipocyte Analysis using ImageJ Software** ....................................................................................... 116

---

x
List of Tables

Table 2.1 European clinical guidelines of cachexia treatments ........................................ 8
List of Figures

Figure 2.1  Stages of cachexia ........................................................................................................ 7
Figure 2.2  Catecholamine regulation of lipolysis ........................................................................... 18
Figure 2.3  Model of IL-6 effects on gene expression ................................................................. 19
Figure 2.4  SNS induced thermogenesis ....................................................................................... 24
Figure 2.5  Browning of white adipose tissue .............................................................................. 25
Figure 3.1  Food intake, body weight, adipose tissue mass and adipocyte size ......................... 43
Figure 3.2  iWAT mass by body weight ......................................................................................... 44
Figure 3.3  Basal and stimulated lipolysis in eWAT and related gene and protein expression ............................................................... 46
Figure 3.4  iWAT protein expression ........................................................................................... 47
Figure 3.5  Energy metabolism in cachectic mice ....................................................................... 51
Figure 3.6  Gene expression in brown adipose tissue ................................................................. 54
Figure 3.7  Gene and protein expression in eWAT ....................................................................... 56
Figure 3.8  Gene and protein expression in iWAT ....................................................................... 57
Figure 3.9  Liver and quadriceps lipid stores ............................................................................... 58
Figure 4.1  Metabolic state, food intake, and body weight differences between Restricted mice and ad-libitum fed Controls ................................................................................. 77
Figure 4.2 Adipose tissue mass and adipocyte size in refeeding Restricted mice by metabolic state and relative to ad-libitum fed Controls.......................................................... 79
Figure 4.3 Liver lipid stores in refeeding Restricted mice by metabolic state and relative to ad-libitum fed Controls.................................................................................. 81
Figure 4.4 Expression of thermogenesis-related genes in brown adipose tissue in refeeding Restricted mice by metabolic state and relative to ad-libitum fed Controls.................. 83
Figure 4.5 Gene expressions in epididymal white adipose tissue in refeeding Restricted mice by metabolic state and relative to ad-libitum fed Controls........................................... 85
Figure 4.6 Hyperinsulinemic-euglycemic clamp measurements of refeeding Restricted mice compared to ad-libitum fed Controls................................................................. 87
Figure B.1 Basal and stimulated lipolysis in mice fasted 24 h........................................... 115
Chapter 1

Introduction

1.1 Introduction

“Your theory is crazy, but not crazy enough to be true” - Neils Bohr

In 2011, using two mouse models of cancer-induced cachexia, Das et al published seminal work demonstrating that preserving fat mass by genetically inhibiting lipolysis also preserved skeletal muscle mass in cachexia (1), a finding that defied computational modeling predictions (2) and conventional wisdom (3) but may have opened new doors to novel therapies for cachexia. Only time will tell if this theory is crazy enough.

Cachexia is defined by most researchers and clinicians as a multi-factorial metabolic disorder characterized by extensive depletion of adipose tissue and skeletal muscle mass that, unlike simple starvation, is remarkably resistant to nutrition therapy (4). Thus, the prominent clinical feature of cachexia is refractory weight loss which affects approximately 30% of cancer patients in Western societies (5). Cachexia is associated with significant morbidity and mortality (5) that is predominantly due to muscle loss (5, 6). Fat loss, however, may precede muscle wasting in cachexia (7) and several recent
longitudinal studies in cancer patients found accelerated loss of adipose tissue predicted poorer survival (8-10). These recent studies, coupled with the seminal work by Das et al, suggest that maintaining fat mass in cachectic patients may improve survival and quality-of-life outcomes.

The mechanisms that promote catabolic events in adipose tissue and skeletal muscle in cachexia are not completely elucidated. Studies have shown reduced adipogenesis (11) and lipogenesis (11, 12), and increased rates of lipolysis (12-18) and lipid utilization (15, 19, 20) are immediate processes promoting loss of adipose tissue in cachexia. However, the mechanisms underlying the increase in lipolysis (catabolism of triglyceride stores) are not completely clear and processes promoting lipid utilization within adipose tissue have been minimally investigated. To better understand these processes and mechanisms, we characterized the enhanced lipolysis and lipid utilization within adipose tissues in cachexia using a murine model of early cancer cachexia. The results of this study are presented in Chapter 3.

A powerful model organism for studying human diseases is the laboratory mouse who shares 99% of its genes with humans and consequently physiological and pathological characteristics (21). Intervention studies using mice may cause a decrease in food intake which confounds interpretation of study results. To assess the effect of a treatment independent of food intake, investigators may provide food to a control group of mice that is equivalent to that consumed by the freely-fed experimentally treated mice, an
experimental strategy known as pair-feeding (22). When pair-feeding involved significant food restrictions in murine studies of cachexia (23, 24) and other dietary interventions in our lab, we intermittently observed changes in feeding behavior in pair-fed mice. To better understand the influence of restricted feeding on feeding behavior and to characterize changes in adiposity that occur during the normal physiological adaptation to calorie restriction, we subjected mice to a short-term food restriction. The results of our observations are presented in Chapter 4.
1.2 Aims

Aim 1: To characterize lipolysis in white adipose tissue during the early stage of cancer-induced cachexia in mice. Increased lipolysis is associated with cancer cachexia but quantitative ex-vivo measurements of lipolysis coupled with metabolic (energy) measurements and investigation of underlying mechanisms have not been conducted. The working hypothesis for this aim is that lipolysis is increased in cancer-induced cachexia as a result of both an energy deficit from reduced food intake and hypermetabolism.

Aim 2: To characterize processes associated with lipid utilization in white and brown adipose tissues during the early stage of cancer-induced cachexia in mice. While whole body lipid oxidation is increased in cachexia, whether markers of fatty acid oxidation in adipose tissue are increased is not well-studied and whether adipocyte thermogenic capacity is altered is unknown. The working hypothesis for this aim is that markers of fatty acid oxidation are increased in cancer-induced cachexia in brown and white adipose tissues and are associated with uncoupling of oxidative phosphorylation and increased oxidative capacity (mitochondrial biogenesis).

Aim 3: To assess feeding behavior and characterize changes in adiposity in mice subjected to a restricted feeding regimen. Mice provided restricted food rations may alter their feeding behavior but intraday effects on lipid metabolism are not well-studied. Characterization of compensatory changes in lipid metabolism in healthy mice on
restricted diets may offer insight into maladaptive responses in cachexia. *The working hypothesis of this aim is that healthy mice metabolically compensate for reduced caloric intake to preferentially maintain adipose stores.*

Chapter 2 provides an overview of the cancer cachexia syndrome followed by a review of the literature pertaining exclusively to catabolic events (lipolysis and lipid utilization) in adipose tissue in cachexia. The second section reviews the mechanisms of lipolysis and lipid utilization in adipose tissue that are specific to the working hypotheses in Aims 1 and 2. The final section of Chapter 2 reviews the effects of food restriction on behavior and adiposity in healthy mice. The basis for this final review was to gain a better understanding of lipid metabolism in the pair-fed control group used in cachexia and other studies.
2.1 Cancer Cachexia

Significant collaborative efforts have been made in the last 5 years to define and stage cancer cachexia – an effort expected to help better assess outcomes in clinical trials and improve treatments in clinical settings. In 2011, a consensus definition was published by an international panel of cachexia researchers and clinicians which defined cancer cachexia as a “multi-factorial syndrome characterized by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment” (25). The panel recognized that cachexia progresses through three stages (Figure 2.1). Diagnostic criteria were proposed: weight loss > 5% (> 2% if BMI < 20) and appendicular skeletal muscle wasting. The consensus definition identifies loss of skeletal muscle as key in a patients’ functional impairment (25), but the prominent features of cachexia also include extensive loss of fat mass (8-10, 26), cytokine-mediated systemic inflammation (27), anorexia (28), and metabolic perturbations (29).
Prevalence, Impact, and Treatments

Cachexia affects about one third of cancer patients and may contribute to up to 20% of cancer deaths (5). Cachexia is associated with reduced tolerance to chemotherapy, chemotherapy toxicity, and poor treatment outcomes (30, 31) and significantly impacts quality-of-life by causing fatigue (32), weakness (33), anorexia (28), and depression (34). Current available treatments are for symptom management and are not curative. A recent consensus rating of nutritional, non-drug, drug, and multi-modal therapies (Table 2.1) highlights the net-benefit and efficacy of treatments currently used to treat symptoms in advanced cancer patients with cachexia.
<table>
<thead>
<tr>
<th>Category</th>
<th>Treatment</th>
<th>Weak Positive</th>
<th>Strong Positive</th>
<th>Weak Negative</th>
<th>Strong Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrition</td>
<td>Enteral Nutrition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parenteral Nutrition</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Supplements*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-drug</td>
<td>Nutrition Counseling</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Physical Training</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Psychotherapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug</td>
<td>Anti-cytokines</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cannabinoids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Omega-3 Fatty Acids*</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Megestrol/Progestins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NSAIDS</td>
<td></td>
<td></td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Prokinetics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Steroids</td>
<td></td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>Multi-modal</td>
<td>Various Combinations</td>
<td>✓</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* insufficient evidence for consensus

Table 2.1 European clinical guidelines of cachexia treatments (35).

Adipose Tissue Depletion in Cancer Cachexia

Loss of fat mass in cachexia is extensive. Studies conducted in the late 1970’s found lung cancer patients lost up to 85% of total body fat (36). In more recent studies, fat losses in patients with cachexia ranged between 35 – 45% (8, 10). Although functional impairment and reduced survival in cachexia are proximately attributed to skeletal muscle loss (25), in recent longitudinal studies of cachectic patients, rate of fat loss predicted poorer survival in advanced lung and colorectal (8) and pancreatic (10) cancer patients, obese cancer patients (9), and in cancer patients in palliative care (26). Importantly, maintaining fat mass may improve survival or quality of life (QoL). In a randomized control trial of patients with advanced gastrointestinal cancers, daily insulin treatments
had significant effects on fat metabolism resulting in accumulation of body fat associated with improved metabolic efficiency and survival (37). In two mouse models of cancer-induced cachexia, preserving fat mass by genetically inhibiting lipolysis also preserved skeletal muscle mass (1) which may improve QoL outcomes.

Metabolic Processes Contributing to Adipose Tissue Depletion in Cachexia

Fat loss from adipose tissue in cancer cachexia is partially, if not primarily (29), the result of increased catabolism of stored triacylglycerols – lipolysis (12-18). In fatty acid kinetic studies in cancer patients, rates of whole body lipolysis as measured by rate of appearance of glycerol were 38 – 70% higher in cachectic patients than in weight-stable healthy subjects (13-15) and 51% higher than weight-stable cancer patients (13). However, whole body lipolysis in cachectic patients was not different than weight-losing controls (14). Investigators using plasma glycerol and non-esterified fatty acid (NEFA) levels as surrogate markers of in vivo lipolytic activity found a 3-fold increase in lipolytic activity in cachectic patients compared to weight-stable cancer patients and healthy controls (16) and a 1.5-fold increase compared to weight-losing cancer controls whose impaired intake alone presumably accounted for this group’s malnutrition (17). In the latter study, in vitro analysis of subcutaneous fat obtained by biopsy also demonstrated increased stimulation of lipolysis with 8-Br-cAMP, a cAMP analogue, in cachectic patients compared to weight-stable and weight-losing cancer controls (17). In murine models of cachexia, in vitro measurements of glycerol release in epididymal fat from cachectic mice bearing a preputial gland tumor showed the basal rate of lipolysis was
enhanced 2-fold but no difference in lipolytic rates was found in epinephrine-stimulated experiments (12). However, epididymal fat from cachectic mice bearing the MAC 16 tumor showed a 3-fold increase in lipolytic response to isoprenaline (18).

Elevated rates of lipolysis in cachexia would contribute to loss of total body fat only if lipid utilization were also increased. Clinical studies measuring $^{13}$CO$_2$ enrichment in breath samples and respiratory quotient (RQ) from indirect calorimetry have demonstrated elevated rates of whole body fatty acid oxidation in weight-losing cancer patients compared to weight stable patients (19, 20) and healthy controls (15). Specific to lipid oxidation in white adipose tissue (WAT), a recent global gene expression profile of subcutaneous adipose tissue from cachectic patients demonstrated genes related to fatty acid degradation, Krebs cycle, and mitochondrial electron transport pathways were highly expressed in WAT of patients that were also found to have low RQ’s (indicating elevated fatty acid oxidation) (20). Finally, analysis of autopsy samples of peri-adrenal tissue revealed brown adipose tissue (BAT) present in 80% of cachectic patients compared with only 13% of controls (29) suggesting increased fatty acid oxidation in adipose tissue secondary to thermogenesis. Only one study in a murine model of cachexia has been conducted that measured whole body fatty acid oxidation, which was increased in tumor-bearing mice (38).

In addition to increased lipolysis and lipid utilization, fat loss in cancer cachexia may also be the result of decreased lipid deposition (lipogenesis) and adipocyte differentiation.
(adipogenesis). In tumor-bearing mice, activity of the lipid uptake proteins (12) and mRNA expression of genes involved in adipocyte differentiation and synthesis (11) were reduced in white adipose tissue.

Mechanisms of Increased Lipolysis in Adipose Tissue in Cachexia

No clear conclusions of the mechanisms promoting increased lipolysis in cancer cachexia have emerged from the few studies performed to date. Reduced energy intake and/or tumor-induced systemic increases in energy expenditure resulting in an energy imbalance (14, 39) may activate canonical pathways (reviewed below) to promote lipolysis. Altered body composition was suggested to also play a role in one fatty acid kinetics study of cachectic patients (14). Other studies in mice and humans have found tumor-induced changes in signaling and enzyme activity that may promote lipolysis within adipose tissue. These changes include a) enhanced expression of the key lipid mobilizing enzyme, hormone sensitive lipase (HSL) (16), b) increased stimulation of β-adrenergic receptors (14, 39) due to catecholamines released in response to stress, c) increased expression of adipocyte Gα-s/Gα-i proteins that would enhance the lipolytic response (18), and d) tumor/adipocyte secretion of the lipid mobilizing protein, zinc-α2 lipoprotein (ZAG) (40, 41).

Mechanisms of Increased Lipid Utilization in Adipose Tissue in Cachexia

Studies in murine models of cachexia suggest sympathetic nervous system activation of brown adipose tissue thermogenesis by cytokines (38, 42) or peripheral activation by
zinc-α2 glycoprotein (ZAG) may enhance lipid utilization within adipose tissue. ZAG is produced locally in BAT and WAT (41) and by some adenocarcinomas (43) and binds with high affinity to β3 adrenergic receptors (44). ZAG administration in mice leads to an upregulation of UCP1 gene and protein expression in BAT (45) – essential to thermogenesis – and an increase in whole body lipid oxidation (40). In addition to thermogenesis as a cause of elevated fatty acid oxidation in adipose tissue, a recent study in humans found the cell death–inducing DNA fragmentation factor-A–like effector A (CIDEA) in cachectic patients may inactivate the pyruvate dehydrogenase complex which would favor the oxidation of fatty acids over glucose in adipose tissue (46).
2.2 Lipolysis

In adipose tissue, the enzymatic hydrolysis of stored triacylglycerol into fatty acids (lipolysis) is a regulated process that maintains body energy and temperature homeostasis in response to physiological conditions. Lipolysis in white adipose tissue provides fatty acids to peripheral tissues in times of negative energy balance (47). Lipolysis in brown adipose tissue – in newborns and small mammals – provides fatty acids for heat production in response to cold stress (48) and brown adipose tissue itself plays a role in energy homeostasis, possibly even in adult humans (49).

Triacylglycerols (TAG) are deposited in a large, unilocular lipid droplet in the cytosol of adipocytes of WAT. In BAT, lipid is deposited in small, multiple separate droplets (multilocular) heavily surrounded by mitochondria. BAT is more highly vascularized than WAT, but both depots are innervated by a large number of noradrenergic fibers (50). In both white and brown adipocytes, the lipid droplet is surrounded by a phospholipid hemi-membrane that is coated with a variety of structural and regulatory proteins, including perilipin (51). Lipolysis of triacylglycerols within this lipid droplet is under hormonal, adipokinal, cytokinal, and neural regulation and is accomplished in consecutive steps by intracytoplasmic lipases aided by lipid droplet-associated proteins (52, 53).

A description and function of the key lipases and lipid droplet proteins mediating lipolysis are described below followed by the known signaling pathways and molecular
mechanisms that regulate lipolysis (both classical and pathological) pertinent to the working hypothesis.

**Lipases**

Three lipases act sequentially to hydrolyze fatty acids from triacylglycerols.

i. Adipose Triglyceride Lipase (ATGL) is responsible for most of the hydrolase activity against TAG in adipocytes, its specific activity against TAG being 10 fold greater than against diacylglycerols (52). ATGL is phosphorylated on serine 404 and 428, but the kinase responsible is unknown and it is unclear how phosphorylation affects this lipase’s activity (52). ATGL deficient mice accumulate excess lipid (TAG) in multiple organs and exhibit cold sensitivity (52) while transgenic mice overexpressing ATGL show elevated lipolysis and subsequent increased fatty acid oxidation in WAT (54). In 3T3-L1 adipocytes, ATGL mRNA levels are negatively regulated by insulin and TNF-α (55) and positively regulated by glucocorticoids (56) and PPARα and PPARγ agonists (52) but the extent to which enzyme activity is affected in vivo is unknown.

ii. Hormone sensitive lipase (HSL) is the primary diacylglycerol (DAG) lipase involved in lipid breakdown. The adipocyte isoform of HSL (84 kDa) preferentially hydrolyzes DAG, its specific activity against DAG being 10 fold greater than against TAG (52). Protein kinase A (PKA) phosphorylates HSL at three serine residues (Ser 563, 659, 660), the latter two being necessary for translocation of HSL to the lipid droplet (57). In 3T3-L1 cells, the extracellular
signal-regulated kinases (ERKs), p42 and p44 mitogen activated protein kinases (MAPKs), phosphorylate HSL at Ser 600 and are themselves activated through β-adrenergic receptors (58). 5’-AMP–activated kinase (AMPK) phosphorylates HSL Ser 565 and has been shown to prevent PKA-mediated phosphorylation and thus potentially inhibit lipolysis (57). HSL deficient mice exhibit blunted stimulated lipolysis and accumulate DAG in adipose tissue (52) while transfected adipocytes overexpressing rat HSL show diminished TAG accumulation presumably due to accelerated lipolysis (59). HSL mRNA expression is negatively regulated by insulin and TNF-α in 3T3-L1 adipocytes (55) and positively regulated by dexamethasone in rat adipocytes (56). In general, HSL is predominantly regulated by post-translational mechanisms (53).

iii. Monoacylglycerol lipase (MGL) performs the final step in lipolysis when it hydrolyzes monacylglycerols producing glycerol and fatty acids. Evidence suggests MGL activity is not regulated hormonally (53).

**Lipid Droplet Proteins**

Perilipin, a protein in the PAT (Perilipin, Adipophilin, TIP47) domain family, is the most abundant lipid droplet-associated protein in adipocytes. Perilipin A (62 kDa), the primary isoform, is tightly anchored to the phospholipid hemi-membrane of the lipid droplet and functions to passively protect triacylglycerols from lipolysis during basal (fed) conditions and actively assist in lipolysis during stimulated (fasted) conditions (60, 61). Murine perilipin has six consensus sites for PKA phosphorylation (Ser 81, 222, 276,
Phosphorylation of one or more of the amino terminals’ RRXS/T motifs (Ser 81, 222, or 276) is required for HSL docking to the lipid droplet and/or HSL access to triacylglycerols and thus is necessary for maximal lipolysis (61). Phosphorylation of Ser 517 or 492 has been found to increase the availability of ATGL’s co-activator, CGI-58, and be required for ATGL activation in 3T3-L1 cells (63). Phosphorylation of Ser 492 in 3T3-L1 fibroblasts triggers fragmentation of the lipid droplet which may facilitate lipolysis by increasing surface area (61). Perilipin deficient mice exhibit increased basal lipolysis (protective “barrier” function is compromised) and decreased catecholamine-stimulated lipolysis (docking and fragmentation function is reduced). Additionally, perilipin null mice are lean but develop peripheral insulin resistance (64). Perilipin gene expression in 3T3-L1 adipocytes is positively regulated by PPARγ and negatively regulated by TNF-α via p44/42 (65).

AB-hydrolase containing 5 (ABHD5), also known as CGI-58, is a lipid droplet associated protein of the α/β hydrolase protein family that binds to perilipin on the lipid droplet in a non-stimulated state and acts as a co-activator of ATGL in the stimulated state – increasing ATGL activity up to 20 fold (53). Recent experiments have shown the interaction of CGI-58 with perilipin and ATGL is mutually exclusive and thus is an important regulatory control point of lipolysis (63).

*Catecholamine Regulation of Lipolysis*
Signaling in this classical pathway (Figure 2.2) is initiated with the binding of catecholamines (epinephrine and norepinephrine) to β-adrenergic receptors ($\beta_1$, $\beta_2$, $\beta_3$) linked to stimulatory G-proteins ($G_\alpha_s$) that activate adenylate cyclase to synthesize and release cyclic (c)AMP. cAMP, in turn, binds and activates protein kinase A (PKA) which induces lipolysis by phosphorylating HSL and perilipin (53). Phosphorylation of HSL at Ser 659/660 results in translocation of HSL to the lipid droplet (57) where perilipin facilitates HSL activity by providing a scaffold for HSL docking to the lipid droplet and/or access to TAG (61). Adipose triglyceride lipase (ATGL)’s co-activator, ABHD5, is made available to ATGL by PKA phosphorylation of perilipin which releases ABHD5 from perilipin (53). Activated ATGL then initiates lipolysis by hydrolyzing TAG to DAG. HSL mediates the hydrolysis of DAG to monoacylglycerol (MAG) and the final enzymatic step in TAG breakdown is completed by MGL (52). Insulin is the primary anti-lipolytic hormone in this pathway. Inhibition of lipolysis is mediated by insulin binding to its receptor, signaling through IRS-1/PI3K, and activating phosphodiesterase 3B (PDE3B) to hydrolyze cAMP to 5’AMP which ultimately inhibits lipolysis (66).
Cytokine Regulation of Lipolysis

Lipolysis may also be regulated by cytokines. When tumor necrosis factor (TNF-α) binds to its adipocyte cell surface receptor, it rapidly activates the extracellular signal related kinase (ERK) signaling pathway. In 3T3-L1 experiments, this activation stimulates lipolysis in the short term through the phosphorylation of HSL (58) and in the long-term through changes in gene expression (68). Activated ERK phosphorylates PPARγ which reduces its transcriptional activation of genes encoding perilipin (68). As a consequence, perilipin mRNA and protein levels in the adipocyte are decreased leaving the lipid droplet exposed to lipases which increases basal lipolysis (64). Additionally, TNF-α
significantly down-regulates phosphodiesterase 3B (PDE3B) activity, protein, and mRNA resulting in a prolonged increase in cAMP levels (69). IL-6 has also been shown to activate the ERK pathway and lead to decreased PPARγ expression and increased lipolysis (70). Whereas TNF-α is thought to act primarily through autocrine and paracrine mechanisms, IL-6 acts through autocrine, paracrine, and endocrine networks (70). A model of effects of ERK activation via IL-6 is presented in Figure 2.3.

Figure 2.3 Model of IL-6 effects on gene expression (70).
Adipokine Regulation of Lipolysis

Zinc-α2-Glycoprotein (ZAG) is a 43 kDa soluble protein expressed and secreted locally in both BAT and WAT (41) and now recognized as a novel adipokine in both humans (71) and mice (41). ZAG is also produced by malignant tumors and secreted into circulation and is identical to a lipid mobilizing factor (LMF) isolated from the urine of cachectic patients (72). The mechanisms by which ZAG may modulate lipolysis are not fully characterized, but well-known is ZAG’s role as a β3-adrenergic receptor agonist. ZAG therefore initiates lipolysis through the classical pathway as described above. ZAG may act locally in an autocrine/paracrine manner to modulate lipolysis (41) but when secreted from malignant tumors acts as an endocrine signal to adipose tissue.
2.3 Lipid Utilization

Following lipolysis, non-esterified fatty acids may be released into systemic circulation to provide energy for peripheral tissues, re-esterified into TAG, or oxidized within adipocytes (47, 66). Oxidation of fatty acids within adipose tissue may result in energy wasting (consequently, avoidable lipid depletion) and is therefore the focus of this dissertation. Possible causes of elevated fatty acid oxidation within adipocytes are re-esterification of fatty acids into TAG due to increased lipolysis and uncoupling of oxidative phosphorylation.

TAG-FA Cycling

Triacylglycerol fatty acid (TAG-FA) cycling occurs when fatty acids released during lipolysis are re-esterified into TAG from DAG or MAG by acyltransferases (14). The energy cost of TAG-FA cycling has been found to be less than 2% of resting energy expenditure in healthy subjects (14) but may substantially increase in pathological and injury states (14, 29) due to increased lipolysis (73). During TAG-FA cycling, AMPK may be allosterically activated (74) due to an increased intracellular AMP/ATP ratio (73) from acylation costs. 5’ AMP activated protein kinase (AMPK), is a 62 kDa heterotrimeric protein kinase (74) that functions as a “sensor of cellular energy state” (73). AMPK functions in the short term to activate ATP producing processes through post-translational modifications (75). Activated AMPK phosphorylates acetyl CoA carboxylase (ACC) decreasing the concentration of malonyl CoA and thereby removing the inhibition on carnitine palmitoyl transferase I (CPT1β) to allow fatty acids to enter the
mitochondria for oxidation (74). AMPK has also recently been shown to increase peroxisome proliferator-activated receptor gamma coactivator (PGC-1α) expression in adipose tissue (76) thereby promoting mitochondrial biogenesis and increasing the oxidative capacity of the adipocyte in the long-term (75, 77).

**Uncoupling of Oxidative Phosphorylation**

Uncoupling of substrate oxidation from ATP synthesis during mitochondrial respiration results in dissipation of energy as heat (thermogenesis) and also increases lipid utilization (48). Uncoupling protein 1 (UCP1) is a 33 kDa protein in the mitochondrial anion carrier family that is responsible for thermogenic capacity and activity in brown and beige adipocytes (78). UCP1 functions to dissipate the proton electrochemical gradient in the mitochondrial inner membrane space thereby uncoupling oxidative phosphorylation and generating heat (79). Brown adipocytes predominate in brown adipose depots while beige adipocytes predominate in white adipose tissue. Classical brown adipocytes are activated by the sympathetic nervous system in response to cold (48) or cytokines (42) while beige adipocytes may be induced and activated under conditions of increased energy expenditure (exercise), PPARγ agonists, β-adrenergic agonists, or cold (78).

**Regulation of Brown and Beige Adipose Tissue Thermogenesis**

Sympathetic nervous system (SNS) activation leads to release of norepinephrine from noradrenergic nerve terminals that innervate BAT and WAT depots (80). Norepinephrine
binds to β-adrenergic receptors ($\beta_1,\beta_2,\beta_3$) linked to stimulatory G-proteins activating adenylate cyclase to synthesize and release cAMP. cAMP, in turn, binds and activates PKA which induces lipolysis (as previously described). Released fatty acids allosterically activate UCP1 thereby uncoupling respiration from ATP synthesis and creating a futile cycle that drives fatty acid utilization (48). PKA also activates p38α MAPK which phosphorylates PPARγ coactivator PGC-1α, a transcriptional coactivator also involved in mitochondrial biogenesis. PPARγ and coactivator bind to the UCP1 peroxisome proliferator response element (PPRE) to stimulate UCP1 gene expression. P38α simultaneously phosphorylates the activating-transcription factor-2 (ATF-2) which also must bind to a cAMP response element (CRE) for UCP1 transcription. Finally, activated ATF-2 stimulates PGC-1α gene expression by binding to CRE2(81) (Figure 2.4). SNS activation of thermogenesis occurs in response to cold stimulus (48), leptin (81), and TNF-α (42).
Figure 2.4 SNS induced thermogenesis (81).

In addition to regulation by sympathetic tone through β-adrenergic signaling, cellular energy sensing may also regulate thermogenic activity (control browning) in white adipose tissue. Recently, based on a review of mouse and human studies, it was proposed that any stimuli that can sufficiently activate the MAPK, AMPK, or PKA signaling pathway in white adipose tissue may induce and activate beige adipocytes (78) (Figure 2.5).
Figure 2.5 Browning of white adipose tissue (78).
2.4 Food Restriction in Mice

Behavioral Response to Food Restriction

Mice fed ad-libitum consume food intermittently (nibble) mostly throughout the dark hours and in short bouts during light hours. In mice housed on a 12 h light/12 h dark cycle, about 70% of food is consumed during the dark hours (22). When food provided to mice is restricted below what would be consumed ad-libitum or merely rationed to levels consumed by weight-matched controls, mice often consume all of the food allotment shortly after it is presented (gorging) instead of nibbling throughout the dark and light hours (22, 82, 83).

Gorging is defined as excessive consumption of food in a short time. In a recent study, mice that gorged in response to food restriction consumed, on average, 38% of their available food in 2 hours (84) at their gorging peak. Mice on a 5% calorie restriction, consumed four times more food in the first two hours of feeding than the ad-libitum fed control group (85). When a mouse ingests all of its food allotment in a short time it is effectively fasted the remaining hours of a 24-hour feeding cycle analogous to mice subjected to time-restricted feeding regimens (22, 86, 87). Pair-feeding in mouse studies, especially if it involves significant food restrictions, triggers gorging behavior (22, 82) and consequently the mouse’s intraday metabolic states include a brief fed state and an extended fasted state until the following day’s food allotment.
Food Restriction Effects on Adiposity

Caloric restriction that creates an energy deficit reduces fat mass. However, due to compensatory reductions in energy expenditure in response to food restriction, mild calorie restriction may increase fat mass in mice (85). Remarkably, in three recent murine studies, mice subjected to mild (5%) and moderate (20%) calorie restrictions had increased or equivalent fat mass respectively compared to ad-libitum fed controls (83-85). In these studies, maintenance and accumulation of fat was favored while lean mass was lost. This preferential accumulation of fat mass is also seen during weight regain. Accelerated rates of fat recovery resulting in excess adiposity have been reported in a rat model of controlled refeeding after semi-starvation (88-91) also attributed to a sustained suppression of thermogenesis (energy expenditure). Thus, food restriction in mice may trigger compensatory metabolic adjustments in energy expenditure that favors fat preservation and/or accumulation.

Compensatory Mechanisms in Response to Food Restriction

Mice and rats that are food restricted reduce energy expenditure (84, 85, 92) but the exact components of the energy budget that are affected remains uncertain (84). In MF1 mice, reductions in locomotor activity and resting metabolic rate accounted for the reduction in total energy expenditure which almost completely compensated for the 20% reduction in energy intake (84). In C57BL/6 mice subjected to a 5% calorie restriction, reductions in resting energy expenditure but not activity accounted for the reduction in total energy expenditure (85). In rats, a significant reduction in resting metabolic rate, beyond what
was expected for change in mass, accounted for the reduction in total energy expenditure (93). In a second study in this rat model, “saved” energy was preferential deposited as lipid in adipose tissue (94), an adaptation proposed to have survival value as fat mass is less energetically expensive (95).

The mechanism by which resting and non-resting energy expenditure is decreased is also uncertain. Reductions in energy expenditure are greater than what would be predicted based on loss of body mass suggesting increased metabolic efficiency. Improved metabolic efficiency (adaptive changes in the efficiency of cellular energy utilization) have been explained by increased skeletal muscle chemomechanical efficiency during activity (96), suppressed SNS-mediated BAT thermogenesis (97), and reductions in body temperature (thus reduced thermoregulatory costs) (98).

**Gorging Effects on Adiposity**

Remarkably, there is a paucity of studies of the relationship between feeding behavior and adiposity in mice that are food restricted. In rat studies of meal patterns, Ozelci et al found that the initial food restriction prior to refeeding was the cause of increased adiposity detected in the rats, not the meal pattern (99). In murine studies, gorging mice were less able to metabolically compensate for reduced food intake due to increased foraging activity – thus gorging reduced adiposity (83). Consistent with this study, mice fed a high fat diet on a time-restricted feeding schedule (gorging) had reduced adiposity compared to ad-libitum fed controls despite similar intakes (100).
Lipogenesis in Response to Food Restriction

During food restriction, researchers have proposed that mice protect fat stores at the expense of lean body mass to reduce their metabolic rate which consequently narrows the gap between intake and expenditure for survival advantage (84, 95). The molecular mechanisms by which fat mass might be preferentially stored or preserved are suggested to be related to muscle insulin resistance and adipose tissue insulin hyperresponsive ness which occurs during re-feeding (89-91) and possibly during restriction (101, 102). Insulin increases both the uptake and synthesis of fatty acids in adipose tissue in rodents. Insulin increases uptake of fatty acids in adipose tissue by post-translationally activating lipoprotein lipase (LPL) (103). Through the induction of the expression of the transcription factor, sterol regulatory element bind protein 1c (SREBP1c), insulin regulates the expression and/or activity of fatty acid synthase (FAS) (104), stearoyl co-A desaturase-1 (SCD1), and acetyl CoA carboxylase (ACC) (105) to promote de novo TAG synthesis. Appropriately, SREBP1c, is considered a “thrifty gene” (106).
Chapter 3

Increased Metabolic Rate and Lipolysis Associated with the Depletion of White Adipose Tissue in Early Cancer Cachexia*

* Kliwer KL, Ke JY, Tian M, Cole RM, Belury MA.
3.1 Abstract

Cancer cachexia is a metabolic disorder characterized by extensive depletion of adipose tissue and skeletal muscle mass. A growing body of literature suggests that maintaining fat mass in cachexia may improve survival outcomes, but the catabolic events promoting adipose depletion have been minimally investigated. In the present study, we characterized lipolysis and processes promoting lipid utilization within adipose tissue in male CD2F1 mice with colon-26 tumor-induced cachexia at the onset of anorexia and weight loss. In this study of early cachexia, adipose tissue mass in cachectic mice was moderately reduced. Total energy expenditure was elevated 9% compared to pair-fed controls despite a 33% reduction in locomotor activity in cachectic mice. Basal (unstimulated) but not isoproterenol stimulated lipolysis was increased in adipose tissue explants of cachectic mice compared to ad-libitum fed controls, but rates were not different than in pair-fed controls. Markers of thermogenesis were increased in brown (but not white) adipose tissue compared to pair-fed controls and uncoupling protein 1 expression paralleled lipolysis rates in tumor-bearing mice. These data suggest that compensatory mechanisms in response to reduced food intake that suppress energy expenditure may be lost in tumor-bearing mice and contribute to the depletion of adipose tissue in cachexia by increasing lipolysis. Hypermetabolism may be explained, in part, by brown adipose tissue thermogenesis.
3.2 Introduction

Cachexia is a multi-factorial metabolic syndrome characterized by marked loss of adipose tissue and skeletal muscle mass (4). Cachexia effects approximately 30% of cancer patients and is associated with significant morbidity and mortality (5) that is predominantly due to muscle loss (5, 6). Fat loss, however, may precede muscle wasting in cachexia (7) and several recent longitudinal studies in cancer patients found accelerated loss of adipose tissue predicted poorer survival (8-10). Moreover, in two murine models of cancer cachexia, Das et al recently demonstrated that preserving fat mass by genetically inhibiting lipolysis also preserved skeletal muscle mass (1). These recent studies suggest that maintaining fat mass in cachectic patients may improve survival and quality-of-life outcomes.

Fat loss from adipose tissue in cancer cachexia is partially, if not primarily, the result of increased lipolysis (12-18), but the underlying mechanisms by which this might occur are still not clear. Elevated rates of lipolysis in cachexia would contribute to loss of total body fat only if lipid utilization were also increased. Indeed, clinical studies have reported elevated rates of whole body fatty acid oxidation in weight losing cancer patients (15, 19, 20). A recent gene expression profile of adipose tissue from cachectic patients demonstrated genes related to fatty acid degradation were highly expressed in patients that also had elevated levels of whole body fatty acid oxidation (20). Additionally, studies in mice and rats suggest increased thermogenesis in brown adipose
tissue (38, 42, 107) contributes to elevated lipid utilization and consequently loss of adipose tissue in cachexia.

The current study seeks to characterize lipolysis and processes promoting lipid utilization within adipose tissue using a murine model of early cancer cachexia. We hypothesized that alterations in adipocyte thermogenic and oxidative capacity may contribute to increased fatty acid oxidation within both brown and white adipose tissue. We anticipated increased lipolysis in early cachexia would be the result of an energy deficit due to both decreased intake and increased energy expenditure.
3.3 Materials and Methods

*Experimental Animals and Study Design.* Five-week-old male CD2F1 mice (BALB/c x DBA/2, Charles River Laboratories, Wilmington, MA) weighing between 19 and 22 grams were randomized by weight on study day 0 into one of three groups: No Tumor (n=10), Tumor (n=10), and Pairfed (n=10). Mice were then injected subcutaneously into the right flank with $1.0 \times 10^6$ colon-26 carcinoma cells suspended in 100 μl PBS (Tumor group) or PBS only (No Tumor, Pairfed groups). Mice were housed individually at a temperature of $22 \pm 0.5^\circ C$ on a 12 h light (0600 – 1800) and 12 h dark (1800 – 0600) cycle and provided free access to water. Mice in the No Tumor and Tumor groups were fed ad-libitum with AIN-93G semi-purified pelleted diet (Research Diets, New Brunswick, NJ). Pairfed mice were rationed the average amount of AIN-93G diet consumed by the Tumor group the day before. On study days 10 and 11, mice were housed in Comprehensive Lab Animal Monitoring System chambers (Columbus Instruments, Columbus, OH) at a temperature of $22 \pm 0.5^\circ C$ on a 12 h light/dark cycle for 24 h of indirect calorimetry measurements. Mice were sacrificed in the fed state on study day 12 (No Tumor, n=5; Tumor, n=10) and day 13 (No Tumor, n=5; Pairfed, n=10). Data from the Pairfed group was shifted back one day to ensure equivalent food intake for metabolic comparisons with Tumor mice. Mice were anesthetized with isoflurane and blood collected by cardiac puncture. Death was ensured by cervical dislocation. Blood was collected in EDTA-treated tubes and then centrifuged at 1500 x g for 20 min at 4°C. Plasma was collected from the upper layer and frozen at -80°C until further analysis. Sections of eWAT were collected for fixation in 4% paraformaldehyde for paraffin
embedding and ex vivo lipolysis assays. Remaining adipose tissues, quadriceps muscle, and liver were excised, weighed, snap frozen in liquid nitrogen, and then stored at -80°C until further analysis. All study procedures were approved by the Institutional Animal Care and Use Committees at The Ohio State University.

Colon-26 Carcinoma Cell Culture. Murine colon-26 (C-26) carcinoma cells were cultured with RPMI 1640 (Invitrogen, Carlsbad, CA) + L-glutamine medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% fetal bovine serum (Invitrogen) and 1% Penicillin-Streptomycin (Invitrogen) at 37°C and 5% CO₂. Cells were harvested at 80% confluence and resuspended in PBS at a concentration of 1.0 x 10⁶ / 100 μl for injection.

Body Weight, Food Intake, and Rectal Temperature Measurements. Body weight, food intake, and rectal temperatures using a Thermalert TH-8 rodent rectal thermometer (Physitemp Instruments, Clifton, NJ) were measured daily between 0830 - 0930.

RT-PCR. Total RNA was extracted from adipose tissue using the RNeasy® Lipid Tissue Mini Kit (Qiagen, Valencia, CA) according to manufacturer directions. RNA concentration was assessed using spectrophotometry by measuring absorbance at 260 nm (A260). RNA was reverse transcribed to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). cDNA was amplified by real-time PCR in an Applied Biosystems 7300 instrument using predesigned primers and probes (TaqMan Gene Expression Assays, Applied Biosystems, Foster City, CA) for target and
housekeeping genes. Target gene expression was normalized to 18S rRNA or GAPDH and expressed as 2-ΔΔCT relative to Control group (108).

Histology. Histology sections (5 µm) of epididymal white adipose tissues from each mouse were mounted on glass slides and stained with hematoxylin and eosin (H&E) by the Histology Core Laboratory, OSU College of Veterinary Medicine for analysis by microscopy. Images were captured using a light microscope (Olympus 1x50 microscope, Olympus, Center Valley, PA) equipped with a Pixera Pro 150ES digital camera (Pixera, Los Gatos, CA). Three to four random microscopic fields per slide were chosen by one evaluator for imaging. ImageJ software (NIH, Bethesda, MD) was used by the same evaluator to quantify the cross-sectional area of 150 adipocytes for each mouse (9 mice per group).

Hepatic and Muscle Lipids. Total lipids were extracted from ~25 and 65 mg sections of livers and muscle respectively from each mouse using the Folch method (109). Lipids were extracted using a 2:1 v/v solution of chloroform and methanol and a 0.88% KCl solution wash. Samples were centrifuged at 1000 g to facilitate phase separation. The organic lower phase was transferred into a pre-weighed tube and then dried under nitrogen. After drying, tubes were reweighed to determine final total lipid weight per sample.
**Ex-vivo Lipolysis Assay.** Epididymal adipose tissue explants (~30 mg) freshly isolated from each mouse were incubated at 37°C in 600 μl of Krebs-Ringer bicarbonate HEPES buffer containing 3% fatty acid free BSA Fraction V (Calbiochem, San Diego, CA) with or without 10 μM isoproterenol (Calbiochem, San Diego, CA). After 3 h of incubation, aliquots were collected and assayed for glycerol content using the Free Glycerol Determination Kit (Sigma-Aldrich). Levels of glycerol were normalized to the weight of the explants (see Appendix A, B).

**Immunoblotting.** Frozen adipose tissues were homogenized in lysis buffer composed of 20 mM Trizma base, 1% Triton-X100, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, and 5 mM Na4P2O7 · 10H2O with the addition of Complete Mini Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Indianapolis, IN) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche Diagnostics, Indianapolis, IN). Homogenates were rocked for 1 h at 4°C and then centrifuged at 16.1k x g for 15 minutes at 4°C. Supernatant was collected and protein concentration was determined by BCA Protein Assay Kit (Pierce, Rockford, IL). Proteins analyzed were separated using sodium dodecyl sulfate polyacrilamide gel electrophoresis and transferred to nitrocellulose membranes using Towbin transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). Membranes were blocked with 5% non-fat dry milk (NFDM) in Tris Buffered Saline with 0.1% Tween-20 (TBST) for 2 h and incubated overnight at 4°C with primary antibodies. After washing, membranes were incubated for 1 h at room temperature with HRP-linked anti-rabbit IgG, (Cell Signaling Technology, Beverly, MA). Bands were detected by
chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Thermoscientific, Rockford, IL) followed by exposure using Carestream Image Station 4000GL PRO (Carestream Molecular Imaging, Woodbridge, CT). Densiometric analysis was conducted using Carestream Molecular Imaging Software. β-actin was used as a loading control to confirm equivalent loading across the gel.

*Indirect Calorimetry.* Six mice from each group were housed individually in Comprehensive Laboratory Animal Monitoring System (Columbus Instruments, Columbus, OH) chambers for metabolic measurements on days 10 - 11 (No Tumor, n=3; Tumor, n=6) and days 11 - 12 (No Tumor, n=3; Pairfed, n=6). Measurements in Pairfed mice were performed the day after Tumor mice to ensure Pairfed mice were provided food rations equivalent to that consumed by the Tumor group during indirect calorimetry. Oxygen consumption rate (VO₂), carbon dioxide production rate (VCO₂), and locomotor activity (beam breaks) were collected every 20 minutes over 24 hours using CLAMS. Food intake was measured manually. Respiratory exchange rate (RER) was calculated as the ratio of VCO₂ to VO₂. Energy expenditure was calculated as calorific value (CV) x VO₂, where CV is 3.815 + 1.232 X RER. To approximate energy balance, absolute energy expenditure was subtracted from the caloric value of food intake for each mouse. To determine the effect of cachexia on energy metabolism, energy expenditure data were analyzed by ANCOVA with relevant covariates.
Statistics. Preplanned tests to detect differences between the Tumor group and controls (No Tumor, Pairfed) were performed using one-way ANOVA with post-hoc Fisher’s test when differences between groups were significant. Analysis of covariance (ANCOVA) was used to analyze metabolic data and to control for effects of body composition/weight. MINITAB 16 (State College, PA), Microsoft Excel (Microsoft Inc., Redmond, WA), and SPSS v21 (IBM, Somers, NY) were used for all data analysis.
3.3 Results

*Colon-26 Carcinoma Reduces White and Brown Adipose Tissue Mass.* Because C-26 carcinoma may induce anorexia in mice (23, 24), we included a pair-fed group in our study to determine effects of the tumor independent of food intake. At study end, cumulative food intake in the Tumor group was decreased 9% compared to the No Tumor group but was not different from Pairfed mice (Figure 3.1A). Likewise, final body weight of Tumor mice was reduced 7.5% compared to the No Tumor group but was not different from the Pairfed group (Figure 3.1B). Despite equivalent food intakes and body weights, epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), and interscapular brown adipose tissue (iBAT) mass were decreased 30, 31, and 26% respectively in Tumor mice compared to Pairfed mice. These losses were even greater when compared to the No Tumor group (eWAT, –34% vs No Tumor; iWAT, –42% vs No Tumor; iBAT, –44% vs No Tumor) (Figure 3.1B). Consistent with eWAT mass, cross-sectional area of epididymal adipocytes was also significantly decreased in Tumor mice compared to Pairfed and No Tumor groups, 28 and 35% respectively, indicating delipidation (Figure 3.1C).

To further examine reductions in fat mass in Tumor mice, we used ANCOVA to analyze the effect of treatment (No Tumor, Tumor, Pairfed) on white adipose tissue mass using tumor free body weight as a covariate. Remarkably, eWAT and iWAT mass were decreased in Tumor mice compared to Pairfed mice but were not different than the No Tumor group by ANCOVA (p < 0.001) indicating fat mass is preserved and/or
accumulates in Pairfed mice which does not occur in Tumor mice. Relationships between body weight (minus tumor weight) and eWAT and iWAT mass are plotted in Figure 3.1D and Figure 3.2 respectively.
Figure 3.1  Food intake, body weight, adipose tissue mass and adipocyte size. (A) Cumulative food intakes, n=10 per group. (B) Body weights and adipose tissue weights at study end, n=10 per group. (C) eWAT adipocyte cross-sectional area, 150 cells/mouse x 9 mice per group with representative images of H&E stained eWAT. Magnification: 200x; Scale Bar: 50 µm. (D) eWAT mass by body weight (minus tumor). eWAT mass in Tumor mice was significantly lower than Pairfed mice but no different than the No Tumor group by ANCOVA with body weight minus tumor weight as a covariate (p < 0.001, post hoc LSD test; n=10 per group). Values represent means ± s.e.m. Data in A, B, and C were evaluated by ANOVA with post-hoc Fisher’s test. $ p < 0.05$, Tumor vs No Tumor; * p < 0.05, Tumor vs Pairfed. eWAT, epididymal white adipose tissue; iWAT, inguinal white adipose tissue; iBAT, interscapular brown adipose tissue.
Figure 3.1

A

Cumulative food intake (g)

Day

0 2 4 6 8 10 12

B

Weight (g)

Body Weight

eWAT  iWAT  iBAT

C

Area (μm²)

eWAT

D

Body Weight – Tumor (g)

eWAT (mg)
Figure 3.2  iWAT mass by body weight. iWAT mass in Tumor mice was significantly lower than Pairfed mice but no different than the No Tumor group by ANCOVA with body weight minus tumor weight as a covariate (p < 0.001, post hoc LSD tests; n=10 per group. iWAT, inguinal white adipose tissue.

Colon-26 Carcinoma Increases Lipolysis. The totality of evidence from human and animal studies suggests elevated lipolysis plays a key role in adipose tissue wasting in cachexia (110). To determine the contribution of lipolysis to reductions in white adipose mass in Tumor mice, we measured basal (unstimulated) and stimulated lipolysis in fresh eWAT explants of all mice in the fed state. Basal lipolysis, expressed as glycerol released per mg of eWAT explant, was significantly higher in Tumor mice compared to No Tumor mice, but was not different from Pairfed mice. In contrast, when explants were stimulated with the beta adrenergic agonist isoproterenol (ISO), glycerol released by Tumor mice was no different than No Tumor and Pairfed groups (Figure 3.3A).
Because there were differences in fat mass among groups, we used ANCOVA to compare basal lipolysis data using fat mass as a covariate. Basal lipolysis in the Tumor group was no different than in No Tumor and Pairfed mice by ANCOVA using eWAT mass or eWAT plus iWAT mass (as an estimate of total body fat) as a covariate. The relationship between basal lipolysis (glycerol released per mg tissue) and eWAT plus iWAT mass for all mice in each group is plotted in Figure 3.3B.

Consistent with the basal (unstimulated) lipolysis assay findings, phosphorylated hormone sensitive lipase (HSL) Ser563, a surrogate marker for eWAT lipolytic activity, was 9-fold higher in Tumor mice compared to No Tumor mice, but was not different from Pairfed mice (Figure 3.3C). Phosphorylated HSL Ser563 from iWAT was 29 and 6-fold higher in Tumor mice compared to No Tumor and Pairfed mice respectively (Figure 3.4). There were no differences in mRNA or protein expression of the key lipases, adipose triglyceride lipase (ATGL) and HSL, between Tumor and control groups (Figure 3.3D) which may explain the equivalent responses (glycerol released) to equivalent stimulus (10 μM isoproterenol).
Figure 3.3 Basal and stimulated lipolysis in eWAT and related gene and protein expression. (A) Unstimulated (basal) and isoproterenol stimulated release of glycerol per mg of eWAT explants, n=9-10 per group. (B) Basal lipolysis by eWAT and iWAT mass. Basal lipolysis in the Tumor group was no different than in No Tumor and Pairfed groups by ANCOVA with eWAT plus iWAT mass as a covariate at a common weight of 848 mg, n=10 per group. (C) Ratio of phosphorylated HSL Ser563 to total HSL with representative blots in eWAT, n=9-10 per group. (D) eWAT mRNA and protein expression with representative blots of ATGL and HSL, n=8-10 per group. Values represent means ± s.e.m. Data in A, C, and D were analyzed using one-way ANOVA with post hoc Fisher’s test. $ p < 0.05, \text{Tumor vs No Tumor}; * p < 0.05, \text{Tumor vs Pairfed}$. ATGL, adipose triglyceride lipase; HSL, hormone sensitive lipase.
Figure 3.4 iWAT protein expression. Ratio of phosphorylated HSL Ser563 to total HSL with representative blots in iWAT, n=9-10 per group. Values represent means ± s.e.m. Data were analyzed using one-way ANOVA with post hoc Fisher’s test. $ p < 0.05$, Tumor vs No Tumor; * p < 0.05, Tumor vs Pairfed. HSL, hormone sensitive lipase.

Finally, in light of our finding of increased basal lipolysis we investigated whether local cytokines, via the extracellular signal response kinase (ERK) 1/2 pathway (58, 70) might play a role in activating lipolysis in this interleukin-6 (IL-6) model of cachexia. IL-6 mRNA expression in eWAT was significantly increased in Tumor mice compared to No Tumor and Pairfed groups, 7 – 10 folds, respectively. We detected no differences, however, in activation of ERK 1/2 in Tumor mice compared to control groups or its ultimate downstream effector perilipin.

Colon-26 Carcinoma Increases Energy Expenditure and Temperature and Reduces Activity. To investigate the extent to which differences in energy expenditure might explain decreased adipose mass in the Tumor group, we utilized indirect calorimetry to
measure metabolic parameters for 24 hours in 6 mice from each group. We first determined the potential contribution of an energy deficit to adipose tissue wasting. Using indirect calorimetry we found 24 h total energy expenditure (TEE) – measured on study days 10 and 11 – was significantly increased in Tumor mice compared to Pairfed mice but was no different than the No Tumor group (Figure 3.5A). Energy balance (energy intake minus TEE) for this 24 h period was significantly decreased in the Tumor group compared to the No Tumor group but no different than the Pairfed group (Figure 3.5A). Next, to determine the effects of the C-26 tumor on energy expenditure, we applied analysis of covariance to calorimetry data using relevant covariates. Body weights were not different among groups during indirect calorimetry (No Tumor, 21.6 +/- 0.6; Tumor, 21.9 +/- 0.4; Pairfed, 21.5 +/- 0.3) and did not affect energy expenditure (p=0.334, ANCOVA). Locomotor activity, however, was significantly lower in the Tumor group compared to No Tumor and Pairfed groups (Figure 3.5B) and tended to affect energy expenditure (p=0.109, ANCOVA). Therefore, to determine the effect of treatment on energy expenditure, we compared energy expenditures using ANCOVA with locomotor activity as a covariate. By ANCOVA, at a common activity of 764 counts, energy expenditure was significantly higher in the Tumor group compared to the Pairfed group but not different than the No Tumor group (Figure 3.5B). A plot of energy expenditure by activity counts every 20 minutes for Tumor and Pairfed mice is presented in Figure 3.5C. Consistent with energy expenditure data, rectal temperatures of Tumor mice taken on the day of indirect calorimetry measurements were significantly increased compared to Pairfed mice but were not different than No Tumor mice. Mean rectal
temperatures in the Tumor group over the 12 days of the study, however, were increased compared to both Pairfed and No Tumor groups (Figure 3.5D).
Figure 3.5  Energy metabolism in cachectic mice. (A) Total energy expenditure, energy intake, and energy balance on study days 10 – 11 (24 h), n=6 per group. (B) Locomotor activity (counts) and energy expenditure analyzed by ANCOVA with activity as a covariate on study days 10 – 11. Results are at a common activity of 764 counts; n=6 per group. (C) Plot of average energy expenditure by average activity count every 20 minutes for Tumor and Pairfed mice. (D) Rectal temperatures on study days 10 - 11 during indirect calorimetry (n=6 per group) and over the 12 days of the study (n=10 per group). Values represent means ± s.e.m. Except when otherwise noted, data were analyzed using one-way ANOVA with post hoc Fisher’s test. $ p < 0.05$, Tumor vs No Tumor; * $ p < 0.05$, Tumor vs Pairfed.
Colon-26 Carcinoma Increases Lipid Utilization. Analysis of calorimetric data showed the respiratory exchange rate (RER) in the Tumor group was significantly lower than mice in the No Tumor group, but no different than the Pairfed group (No Tumor, 0.86 +/- 0.02; Tumor, 0.79 +/- 0.01, Pairfed, 0.80 +/- 0.00). Based on RER values, 70% of the Tumor and Pairfed group’s total energy was derived from lipid whereas 48% of total energy was derived from lipid in the No Tumor group.

We next investigated markers of fatty acid uptake and utilization by metabolically active BAT. In iBAT, mRNA expression of the fatty acid uptake enzyme, lipoprotein lipase (LPL), was increased in Tumor mice compared to No Tumor mice. Expression of the key lipid uptake and transporter protein [fatty acid translocase, cluster of differentiation (CD36)] and genes involved in promoting fatty acid oxidation [peroxisome proliferator activated receptor δ (PPARδ)] and mitochondrial oxidative phosphorylation [cytochrome c oxidase subunit 4 (COX4), cytochrome c oxidase subunit 8b (COX8B)] were increased in Tumor mice compared to Pairfed mice but were no different than the No Tumor group. Glycerokinase (GYK), the key kinase involved in maintaining adequate stores of triglycerides for oxidation in BAT (111) was also significantly increased in Tumor mice compared to both Pairfed and No Tumor groups (Figure 3.6A). Previous studies have suggested brown adipose thermogenesis is associated with wasting in cachexia (38, 42, 107) which would explain increased lipid uptake and utilization in iBAT. mRNA expression of the key kinase (p38α MAPK), transcriptional co-activators [peroxisome proliferator activated receptor gamma coactivator 1α (PGC1α), PR domain zinc finger
protein 16 (PRDM16)], activating enzyme [Type II iodothyronine deiodinase (DIO2)] and uncoupling protein [uncoupling protein 1 (UCP1)] contributing to thermogenic capacity were significantly increased in Tumor mice compared to Pairfed mice but were no different than the No Tumor group with the exception of DIO2 (Figure 3.6B). There were no differences in protein expression of UCP1 (normalized to β-actin) between the Tumor and control groups. However, significant correlations were found between UCP1 protein expression and rectal temperatures ($r = 0.64$, $p < 0.05$) and basal lipolysis ($r = 0.68$, $p < 0.05$) in the Tumor group.
Figure 3.6 Gene expression in brown adipose tissue. (A) mRNA expression of markers of fatty acid uptake and utilization in iBAT, n=8-10. (B) mRNA expression of genes related to brown adipose thermogenesis in iBAT, n=9-10. Values represent means ± s.e.m. Data were analyzed using one-way ANOVA with post hoc Fisher’s test. $ p < 0.05, $ Tumor vs No Tumor; * p < 0.05, Tumor vs Pairfed.
Next, we investigated whether markers of fatty acid oxidation were altered in white adipose tissue. In eWAT, mRNA expression of the transcription factor involved in promoting fatty acid oxidation, PPARδ, was increased in Tumor mice compared to Pairfed mice but was no different than the No Tumor group. The downstream target of PPARδ that promotes beta oxidation, acyl COa oxidase 1 (ACOX1), was significantly increased in the Tumor group compared to both control groups. No significant differences were detected, however, in CPT1β or the mitochondrial cytochrome c oxidase subunits, COX4 or COX5a, between Tumor and Pairfed or No Tumor groups (Figure 3.7A). Given the increased expression of genes related to fatty acid oxidation in Tumor mice, we measured expression of genes associated with adaptive thermogenesis. mRNA expression of the key kinase (p38α MAPK) and activating enzyme (DIO2) involved in promoting the expression of UCP1 were increased in Tumor mice compared to both control groups (Figure 3.7B). However, no differences in mRNA expression of the thermogenic regulatory proteins PGC1-α or PRDM16 (Figure 3.7B) or protein expression of UCP1 (Figure 3.7C) were detected between the Tumor group and control groups. Similar results were found for iWAT (Figure 3.8).
Figure 3.7  Gene and protein expression in eWAT. (A) mRNA expression of markers of fatty acid utilization in eWAT, n=8-10 per group. (B) mRNA expression of genes related to thermogenesis in eWAT, n=9-10 per group. (C) Protein expression of UCP1 in eWAT. Values represent means ± s.e.m. Data were analyzed using one-way ANOVA with post hoc Fisher’s test. $ p < 0.05, Tumor vs No Tumor; * p < 0.05, Tumor vs Pairfed.
Figure 3.8 Gene and protein expression in iWAT. (A) mRNA expression of genes related to thermogenesis in iWAT. (B) Protein expression of UCP1 in IWAT. Values represent means ± s.e.m. Data were analyzed using one-way ANOVA with post hoc Fisher’s test. $ p < 0.05$, Tumor vs No Tumor; * $ p < 0.05$, Tumor vs Pairfed.

Finally, we investigated whether lipids were stored ectopically. Total lipids in both liver and quadriceps muscle in the Tumor group were not different than in Pairfed and No Tumor groups (Figure 3.9).
Figure 3.9  Liver and quadriceps lipid stores. Total hepatic lipids and total lipids in quadriceps muscle, n=10 per group. Values represent means ± s.e.m. Data were analyzed using one-way ANOVA with post hoc Fisher’s test.
3.5 Discussion

The mechanisms that promote catabolic events in adipose tissue in cachexia are not completely elucidated. In the present study, we used the C-26 murine model of cancer-induced cachexia to characterize lipolysis and processes associated with lipid utilization within adipose tissue in early cachexia. Compensatory mechanisms in response to reduced food intake that suppress energy expenditure were absent (or diminished) in tumor-bearing mice in early cachexia. Increased energy expenditure in cachectic mice was accompanied by elevated levels of markers of BAT thermogenesis, whole body lipid oxidation, and elevated lipolysis in white adipose tissue. Taken together, this suggests hypermetabolism contributes to white adipose tissue depletion in early cachexia. Elevated energy expenditure may partially be explained by BAT thermogenesis.

Consistent with other studies of cancer-induced cachexia (7, 11, 112), absolute mass of white adipose tissues in cachectic mice in our study were significantly reduced compared to both ad-libitum fed and pair-fed control groups. Histological analysis suggested the mass differences were due to lipid loss. After controlling for body weight (minus tumor mass), we found both intra-abdominal (eWAT) and subcutaneous (iWAT) fat mass in cachectic mice were proportionally similar to ad-libitum fed controls and significantly reduced only compared to pair-fed controls. Thus, metabolic adjustments in response to reduced food intake did not occur in cachectic mice to the same degree as in healthy mice. Mammals, including humans, compensate for reduced caloric intake (and simultaneously defend fat stores) by increasing digestive efficiency, reducing resting
energy expenditure, and/or reducing activity (83-85, 113). Although cachectic mice significantly reduced activity in our study compared to both control groups, total energy expenditure in cachectic mice still remained elevated compared to pair-fed mice. After controlling for activity, this difference in energy expenditure was even more pronounced. While it is possible that differences in muscle work efficiency in cachectic and pair-fed mice contributed to the detected differences in total energy expenditure (114, 115), these data strongly suggests that resting energy expenditure in cachectic mice is abnormally elevated.

Elevated total and resting energy expenditure has been reported in both human and rodent studies of cachexia (15, 38, 39, 116). Several mechanisms have been suggested to be responsible for this increase, including futile cycling, systemic inflammation, adrenergic stimulation, and brown adipose tissue thermogenesis [reviewed in (117)]. In the present study, we show increased expression of genes related to fatty acid uptake and oxidation, adaptive thermogenesis, and mitochondrial oxidative phosphorylation in iBAT of cachectic mice compared to pair-fed controls which was accompanied by an increase in rectal temperature. Our tumor-bearing mice varied by cachectic “stage” at sacrifice – e.g., loss of white adipose tissue mass relative to pair-fed mice ranged from 9 to 72% – and we detected similar variability in protein expression of UCP1 in iBAT. Thus, we found no difference in UCP1 protein expression in cachectic mice as a group compared to control groups, but expression levels correlated with temperature and the two mice with the most extensive fat loss had the highest protein expression of UCP1. Our results
are consistent with Tsoli et al who found anorectic C-26 mice had elevated energy expenditure accompanied by elevated BAT temperatures with increased UCP1 protein expression (38). Our observations of metabolic and transcript levels, coupled with paralleled increase in basal lipolysis, suggest brown adipose tissue thermogenesis may play an early role in the depletion of white adipose tissue in cachexia.

Although data from our study and others (38, 42, 107) suggests BAT thermogenesis is involved in adipose tissue wasting in cachexia, contrary to our hypothesis, we did not find evidence of “browning” of white adipose tissues in early cachexia. In theory, any signal that can sufficiently stimulate protein kinase A (PKA), mitogen activated protein kinase (MAPK), or AMP activated protein kinase (AMPK) pathways could induce beige adipocytes with thermogenic capacity (browning) in white adipose tissue [reviewed in (78)]. While markers of fatty acid oxidation were increased in eWAT, markers of thermogenesis and mitochondrial electron transport were mostly unchanged (at times, reduced) in white adipose depots in our study of early cachexia. This contrasts with findings in late cachexia in the MAC-16 model of cachexia, where morphological analysis of epididymal adipose tissue revealed remodeling reminiscent of brown-type adipocytes which was accompanied by increased mRNA expression of PGC-1α (11). We sacrificed C-26 tumor bearing mice at the onset of anorexia and weight loss, perhaps too early for sufficient stimulation to induce beige adipocytes. We did however find that markers of fatty acid oxidation within white adipose tissue were increased, resembling
findings in humans (20) which was suggested to be a result of a substrate switch to fatty acids (46).

To our knowledge, we are the first to use a murine model of cachexia to measure rates of lipolysis considering energy expenditure, affects of adiposity, and potential molecular mechanisms. Similar to findings in mice bearing a preputial gland tumor (12), we found unstimulated lipolysis was enhanced (~1.5 fold) in cachectic mice but stimulated rates were unchanged compared to ad-libitum fed controls. The basal (unstimulated) rate, however, was similar to pair-fed mice. Our energy balance data (measured one day prior to lipolysis assays) showed energy balance in pair-fed and cachectic mice was similar which may explain equivalent basal lipolytic rates in response to energy demand. Furthermore, neither pair-fed nor cachectic mice accumulated lipids ectopically (in liver or quadriceps muscle) suggesting hydrolyzed fatty acids were oxidized to meet energy demands. That basal lipolysis was no different than ad-libitum fed controls after adjusting for fat mass is in line with recent research showing that adiposity may modulate lipolytic rate with a consequent inverse relationship between lipolytic rate per unit of fat mass and total body adiposity (118). Our results also mirror one of the few kinetic studies in human cancer patients which concluded that semi-starvation (i.e. energy imbalance) and/or changes in body composition were responsible for increased lipolytic rates in cachectic patients (14). Finally, although energy balance between pair-fed and cachectic mice was similar in the 24 h of our measurements, the cumulative effects of the small (non-significant) difference may explain differences in total body adiposity. Nonetheless,
given the stark difference in fat mass between cachectic and pair-fed mice, our data also suggests that impairment of lipid uptake and storage may play a significant role in adipose tissue depletion in early cachexia.

In summary, in the present study we observed lipolysis is increased in early cachexia which may be partially explained by an energy deficit resulting from an increase in total energy expenditure accompanied by a reduction in food intake. Brown adipose tissue thermogenesis may contribute to the hypermetabolism and whole body fatty acid oxidation. That tumor-bearing and pair-fed mice had similar lipolytic rates but significant differences in adipose tissue mass suggests future studies should examine the contribution of impairment in lipid storage to adipose depletion in cancer cachexia.
3.6 Acknowledgements

This work was supported by the Pelotonia Graduate Fellowship Program and the Kennedy Professorship at The Ohio State University.
Chapter 4

Short-term Food Restriction with Controlled Refeeding Triggers Gorging Behavior,
Enhances Fat Deposition, and Diminishes Insulin Sensitivity in Mice*

* Kliewer KL, Ke JY, Cole RM, Stout MB, Samuel VT, Shulman GI, Belury MA.
4.1 Abstract

**Background:** Rodents are commonly used in food restriction-refeeding studies to investigate weight regain. Mice that are food restricted and subsequently refed may consume all allocated food in a short time (gorge) which contrasts with nibbling feeding behavior in mice fed ad-libitum. Consequently, the mice that gorge undergo a brief “fed” and extended “fasted” metabolic states during each 24-hour feeding cycle.

**Objectives:** To determine the intraday metabolism-related changes in restriction-triggered gorging mice during weight regain.

**Methods:** Mice were food restricted for 5 days to trigger gorging and subsequently refed at levels similar to intakes of age-matched controls. Lipid stores and expression of genes involved in adipose tissue metabolism and inflammation were measured in gorging mice during their daily fed and fasted metabolic states and compared to ad-libitum fed mice. The hyperinsulinemic-euglycemic clamp was used in a second study to investigate insulin sensitivity during refeeding.

**Results:** Food restriction triggered gorging behavior that persisted when mice were refed controlled rations. Despite reduced cumulative food intake compared to ad-libitum fed mice, intra-abdominal fat mass was increased in restriction–triggered gorging mice but did not vary by metabolic state. Robust intraday changes in adipose tissue lipogenic and inflammatory gene expression were found in gorging mice. Lipogenic gene expression,
however, was elevated and uncoupling protein 1 gene expression was suppressed
throughout the feeding cycle compared to ad-libitum fed mice. Whole-body glucose
disposal was decreased and basal endogenous glucose production was increased in
restriction-triggered gorging mice.

Conclusion: Intra-abdominal fat accumulation is enhanced despite controlled refeeding
in restriction-triggered gorging mice which may be partially explained by suppressed
thermogenic capacity and a gene expression profile favoring lipid deposition despite
intraday fluctuations in gene expression. Importantly, our study demonstrates diminished
insulin sensitivity in restriction-triggered gorging mice during weight regain.
4.2 Introduction

The majority of individuals that lose weight are unable to maintain the weight loss over time (119). Adipose tissue lipid stores are efficiently repleted with weight regain (113) and a disproportionate regain in fat mass relative to lean tissue has been observed in postmenopausal women (120) and the elderly (121). Similarly, preferential fat accumulation has been observed during catch-up growth in low-birth-weight infants (122) and following starvation (123).

Rodent models are commonly used to elucidate the biological mechanisms promoting adipose tissue regain after weight loss and accelerated fat deposition after growth arrest (88-92, 94, 99, 124-127). In these “refeeding” studies, rodents are calorie/food restricted to induce weight loss or arrest growth and subsequently refed at levels equivalent to intakes of matched controls or provided ad-libitum access to food. Energy utilization or expression and activity of genes and proteins involved in lipid metabolism are then examined to characterize the mechanisms promoting fat accumulation after cessation of food restriction.

A potential confounding factor in rodent refeeding studies is the effect of feeding behavior on gene and protein expression, whole-body metabolism, and lipid stores. When daily food rations are restricted below what would be consumed ad-libitum (e.g. food restriction) or rationed to levels consumed by matched controls (e.g. refeeding), rodents may exhibit a gorging pattern of food intake where all allotted food is ingested shortly
after it is presented (22, 82, 83). This feeding behavior contrasts with the nibbling pattern of food intake in ad-libitum fed rodents where food is consumed intermittently mostly throughout the dark hours and in short bouts during light hours (22). As a result of these eating patterns, the daily metabolic states of gorging rodents on a rationed diet include a brief fed state and an extended fasted state until the following day’s food allotment (22) which contrasts with nibbling rodents that effectively remain in a constant fed state. Consequently, metabolism-related gene and protein expression levels, metabolic responses, and lipid stores in gorging rodents may vary considerably in a daily feeding cycle and be at extremes immediately before and after feeding.

To our knowledge, no study has comprehensively examined intraday metabolism-related changes consequent to restriction-triggered gorging during weight regain. Accordingly, in the current studies, we measured lipid stores, metabolites and hormones, and expression of genes involved in adipose tissue metabolism and inflammation in restriction-triggered gorging mice during their daily fed period (gorging) and at the end of their daily fasted period. Additionally, using hyperinsulinemic-euglycemic clamps, we investigated insulin sensitivity in restriction-triggered gorging mice. Our aims were to capture intraday oscillations in lipid stores and adipose tissue gene expressions and to identify metabolic consequences of food restriction-triggered gorging during weight regain.
4.3 Materials and Methods

*Animals and Study Design.* Two studies, almost identical in design, were used to meet the objectives of this paper. The primary differences between the studies were location of housing and data collected (see subsequent paragraphs). Study 1 was conducted at The Ohio State University (OSU) and Study 2 at Yale University. All study procedures were approved by the Institutional Animal Care and Use Committees at the respective institutions.

In study 1, five-week-old male CD2F1 mice (BALB/c x DBA/2, Charles River Laboratories, Wilmington, MA, USA) were randomized by weight into two groups: Control (n=10) and Restricted (n=20). Mice were individually housed at a temperature of 22 ± 0.5°C on a 12:12h light-dark cycle and provided free access to water. Control mice were fed ad-libitum with AIN-93G semi-purified pelleted diet (Research Diets, New Brunswick, NJ, USA). Restricted mice were rationed AIN-93G diet early in the light cycle. Food rations were restricted ~50% relative to Control mice over the first 5 days of the study and approximated Control group intake the remaining 13 days. This feeding regimen was based on a pair-feeding schedule that resembles food provisions in refeeding studies and is known to trigger gorging in mice (unpublished observation). Mice were sacrificed after 18 days. Control mice were fed ad-libitum until sacrifice. Refed Restricted mice gorge all allotted food shortly after presentation and therefore undergo a brief fed period and an extended fasted period every 24 h feeding cycle (Figure 4.1A). One half the Restricted mice were sacrificed at the end of the extended fast and the other
half were sacrificed ~3–4 h after food provision (during gorging). These groups were designated Restricted (Post-gorging) and Restricted (Gorging) respectively. Mice were anesthetized with isoflurane and blood was collected by cardiac puncture into EDTA-treated tubes. Tubes were centrifuged at 1500 x g for 20 min at 4°C and plasma was collected and frozen at -80°C until analysis. Tissues were excised, weighed, snap frozen in liquid nitrogen and stored at -80°C until analysis except sections of epididymal white adipose tissue (eWAT) that were fixed in 4% paraformaldehyde for paraffin embedding.

In study 2, five-to-seven-week-old male CD2F1 mice (BALB/c x DBA/2, Charles River Laboratories) were randomized by weight into two groups: Control (n=12) and Restricted (n=12). Housing (except location), diet, and feeding schedule were the same as study 1. Food for Restricted mice was restricted ~50% compared to Control mice over the first 5 days of the study and approximated Control group intake (81-106%) until mice were clamped on days 14–17. Tissues were collected as described in the clamp procedures.

*Food Intake, Body Weight, and Behavior.* Food intake and body weight were measured daily early in the light cycle. In study 1, feeding behavior was observed when food was presented and cages of Restricted mice were observed daily near the end of the light cycle for food remnants to confirm gorging behavior. In study 2, gorging in Restricted mice was noted intermittently during the study.
**Quantitative Real-time PCR.** Total RNA was extracted from adipose tissue in study 1 using the RNeasy® Lipid Tissue Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer directions. RNA was reverse transcribed to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). cDNA was amplified by real-time PCR in an Applied Biosystems 7300 instrument using predesigned primers and probes (TaqMan Gene Expression Assays, Applied Biosystems). Target gene expression was normalized to 18S rRNA and expressed as $2^{-\Delta\Delta CT}$ relative to Control groups.(108)

**Histology.** Sections of eWAT (5 µm) from each mouse in study 1 were mounted on glass slides and stained with hematoxylin and eosin (H&E) by the Histology Core Laboratory, OSU College of Veterinary Medicine. Images were captured using a light microscope (Olympus, Center Valley, PA, USA) equipped with a Pixera Pro 150ES digital camera (Pixera, Los Gatos, CA, USA). Three to five fields per slide were randomly chosen by one blinded evaluator for imaging. Images were analyzed using ImageJ software (NIH, Bethesda, MD, USA) to measure cross-sectional area of 150 randomly chosen adipocytes per mouse (Appendix C).

**Liver Lipids.** Total lipids were extracted from ~50 mg sections of livers from each mouse in study 1 using the Folch method.(109) Lipids were extracted from samples using a 2:1 v/v solution of chloroform and methanol and a 0.88% KCl solution wash. Samples were centrifuged at 1000 x g to facilitate phase separation. The organic lower phase was
transferred into a pre-weighed tube and dried under nitrogen. After drying, tubes were reweighed to determine total lipid weight per sample.

**Plasma Measurements.** Plasma leptin and adiponectin were measured by ELISA (Millipore, Billerica, MA, USA) according to manufacturer instructions. Plasma β-hydroxybutyrate was quantified by colorimetric assay (Sigma Aldrich, St. Louis, MO, USA).

**Hyperinsulinemic-euglycemic Clamp.** In study 2, indwelling catheters were placed into the internal jugular vein of mice 7–8 days prior to clamp experiments. Mice were fasted overnight, a standard procedure to assess glucose utilization during clamp experiments.(128) Restricted mice, however, had consumed all daily rations prior to food removal for fasting. Thus, the eating pattern and extended fast of Restricted mice were undisturbed by the clamp experiments. Basal whole-body glucose turnover was measured by infusing [3-3H]-glucose at a rate of 0.05 μCi/min for 120 min. After basal measurements, the hyperinsulinemic-euglycemic clamp was conducted for 140 min with a 4-min primed infusion (20 mU/kg) followed by continuous infusion (3 mU/kg/min) of human insulin (Novolin R, Novo Nordisk, Princeton, NJ, USA). [3-3H] glucose was continuously infused at 0.1 μCi/min and a variable infusion of 20% dextrose was used to maintain euglycemia (~120 mg/dl). Plasma samples were obtained from the tail at set time points. 2-deoxy-d-[1-14C] glucose was injected as a bolus (10 μCi) at 85 min to measure tissue-specific glucose uptake. At study completion, mice were anesthetized
with pentobarbital sodium injection (150 mg/kg) and tissues were excised, snap-frozen in liquid nitrogen, and stored at −80°C until analysis.

*Clamp Biochemical Analysis and Calculations.* Plasma glucose was measured using a YSI 2700D glucose analyzer (YSI Life Sciences, Yellow Springs, OH, USA). Plasma insulin was determined by radioimmunoassay kit (Millipore). To measure plasma $^3$H-glucose and 2-deoxy-d-[1-$^{14}$C]-glucose, plasma was deproteinized with ZnSO$_4$ and Ba(OH)$_2$, dried to remove $^3$H$_2$O, resuspended in water, and counted in scintillation fluid using a Beckman Coulter (Brea, CA, USA) scintillation counter. Glucose turnover rates were calculated as the ratio of the [3-$^3$H]-glucose infusion rate to the specific activity of plasma glucose at the end of the basal period and during the final (on average) 30 min of steady state of the clamp. Endogenous glucose production was determined by subtracting glucose infusion rate from whole-body insulin-stimulated glucose disposal. Basal hepatic insulin resistance index was calculated as basal endogenous glucose production × fasting plasma insulin.(129) To measure tissue-specific glucose disposal, tissue samples were homogenized and supernatants subjected to an ion-exchange column (Bio-Rad, Hercules, CA, USA) to separate tissue 2-deoxy-d-[1-$^{14}$C]-glucose-6-phosphate from 2-deoxy-d-[1-$^{14}$C]-glucose. Tissue glucose uptake was calculated from area under curve of plasma 2-deoxy-d-[1-$^{14}$C]-glucose and tissue 2-deoxy-d-[1-$^{14}$C]-glucose-6-phosphate content.

*Statistics.* Data are expressed as means ± s.e.m. Statistical analyses consisted of two-tailed unpaired Student’s t-test, Mann-Whitney test, one-way ANOVA with *post-hoc*
Tukey’s test, or ANCOVA as noted in figure legends. Data not meeting test assumptions were transformed for analysis as indicated in figure legends; however, un-transformed means are presented for relevance. MINITAB 16 (State College, PA, USA), Microsoft Excel (Microsoft Inc., Redmond, WA, USA), and SPSS v21 (IBM, Somers, NY, USA) were used for data analysis. P-value < 0.05 was considered significant.
4.4 Results

*Food Intake, Behavior, and Body Weight.* Over the first 5 days of study 1, Restricted mice were rationed and consumed ~50% less food than ad-libitum fed Control mice. After day 5, food intake of Restricted mice ranged from 98 to 122% of Control group intake (Figure 4.1B). Cumulative food intake was decreased in Restricted mice (Control, 51.0 ± 1.1 g; Restricted, 45.6 ± 0.3 g, p < 0.01).

Mice were fed between ~0830–1000 h daily and Restricted mice had consumed all rations when cages were observed between 1630–1730 h. For necropsy, Restricted (Gorging) mice were fed at 0730 h and were observed during necropsy (~3–4 h later) to have fully distended stomachs filled with food lending support that gorging behavior persisted until study end.

At the start of study 1, Restricted and Control groups had similar body weights. During the 5 days of food restriction, Restricted mice lost 21% body weight compared to Control mice. After day 5, Restricted mice began gaining weight and by study’s end body weight of Restricted mice was 4% lower than Control mice (p < 0.05) (Figure 4.1C).
Figure 4.1 Metabolic state, food intake, and body weight differences between Restricted mice and ad-libitum fed Controls. (A) Metabolic states of Control and Restricted mice in 24 h feeding cycle. Restricted mice gorge all food rations shortly after presentation and therefore experience a brief “fed” period and an extended “fasted” period until food is provided the following day. Control mice nibble throughout dark hours and in short bouts during light hours and therefore effectively remain in a constant “fed” state. (B) Food intakes and (C) Body weights during food restriction (first 5 days) and refeeding. Values represent means ± s.e.m. (Control, n=10; Restricted, n=20). Food intakes were evaluated by Mann-Whitney test and body weights by Student’s t-test. Asterisks indicate statistically significant differences, p < 0.05.
Adipose Tissue Mass and Adipocyte Size. To determine the extent to which adipose lipid storage is affected by a temporary food restriction and intraday changes in metabolic state consequent to gorging during refeeding, we compared adipose depots of Restricted mice during gorging and at the end of their extended fast. eWAT mass of Restricted (Gorging) and Restricted (Post-gorging) mice were significantly higher, 21 and 31% respectively, than in Control mice and were not different from each other (Figure 4.2A). A plot of eWAT mass by body weight for individual mice is presented in Figure 4.2B. Consistent with eWAT weights, cross-sectional area of epididymal adipocytes of Restricted (Gorging) and Restricted (Post-gorging) mice were also significantly higher, 31 and 27% respectively, than in Control mice and were not different from each other (Figure 4.2C, D). Inguinal fat pad weights were not significantly different among groups (Figure 4.2A).
Figure 4.2  Adipose tissue mass and adipocyte size in refeed Restricted mice by metabolic state and relative to ad-libitum fed Controls. (A) White adipose tissue weights, n=10 per group. (B) eWAT mass by body weight. eWAT mass was significantly higher in both Restricted groups compared to Control mice by ANCOVA with body weight as a covariate (p < 0.001, post hoc LSD tests; n=10 per group). (C) eWAT adipocyte cross-sectional area, 150 cells/mouse x 8–10 mice per group. (D) Representative images of H&E stained eWAT revealing effects of restriction-triggered gorging on adipocyte morphology. Magnification: 200x; Scale Bar: 50µm. Data in A and C were analyzed using one-way ANOVA with post hoc Tukey’s test. Different letters indicate statistically significant differences among groups, p < 0.05. eWAT, epididymal white adipose tissue; iWAT, inguinal white adipose tissue.
Intraday Changes in Liver Lipid Stores. Given that fasting may induce hepatic steatosis (130), we measured hepatic lipids in all groups. Total liver lipids were ~24% higher in Restricted (Post-gorging) mice compared to Control and Restricted (Gorging) groups, which were not different from each other (Figure 4.3A). To gauge the degree of fasting in each group, we measured plasma β-hydroxybutyrate, the major ketone formed during fasting. Plasma β-hydroxybutyrate levels were 213% higher in Restricted (Post-gorging) mice compared to Control mice (ANOVA, p=0.081) (Figure 4.3B).
Figure 4.3  Liver lipid stores in refed Restricted mice by metabolic state. (A) Total liver lipids (n=9–10 per group). (B) Plasma β-hydroxybutyrate (n=4–7 per group) was measured to assess degree of fasting. Values represent means ± s.e.m. Data were analyzed using one-way ANOVA with post hoc Tukey’s test. Different letters indicate statistically significant differences among groups, p < 0.05.
*Intraday Changes in Brown Adipose Tissue Gene Expression.* Brown adipose tissue (BAT) thermogenesis is accomplished by uncoupling protein 1 (UCP1) which uncouples mitochondrial respiration from ATP synthesis leading to heat production (thereby promoting energy expenditure) (131). To determine whether BAT thermogenic capacity is altered by restriction-triggered gorging, mRNA expression of thermogenesis-related genes were measured in interscapular BAT (iBAT) in all groups. Expression of UCP1 was significantly decreased in both Restricted groups compared to Control mice. Gene expression of peroxisome proliferator activated receptor gamma coactivator 1α (PGC1α), a transcriptional co-activator crucial to thermogenesis (132), was also significantly decreased in Restricted (Gorging) mice relative to Control mice. There were no significant differences in expression of either gene between Restricted groups (Figure 4.4).
Figure 4.4 Expression of thermogenesis-related genes in brown adipose tissue in refed Restricted mice by metabolic state. Values represent means ± s.e.m., n=7–10 per group. Data were analyzed using one-way ANOVA with post hoc Tukey’s test. Different letters indicate statistically significant differences among groups, p < 0.05.

*Intraday Changes in Lipid Metabolism-related Gene Expression.* Because genes promoting lipid storage and mobilization would likely be affected by consumption of a large amount of food and/or an extended fast, we measured expression of genes involved in adipo/lipogenesis and lipolysis in all groups. Gene expression of key enzymes and transcription factors promoting *de novo* lipogenesis [fatty acid synthase (FAS), sterol-regulatory element binding protein-1 (SREBP-1), stearoyl-CoA desaturase 1 (SCD1)], adipogenesis [peroxisome proliferator activated receptor γ (PPARγ)], and lipid uptake [lipoprotein lipase (LPL)] in eWAT were significantly higher in Restricted (Gorging) mice compared to Control mice. With the exception of SCD1, mRNA levels of these genes were significantly decreased in Restricted (Post-gorging) mice compared to
Restricted (Gorging) mice. Of interest, expression of FAS and LPL were not different and SCD1 was increased in Restricted (Post-gorging) compared to Control mice (Figure 4.5A). No differences in gene expression were detected among groups for adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), or the lipid droplet protein perilipin (PLIN1), which play a role in regulating lipolysis (Figure 4.5A).

Intraday Changes in Adipokine Gene Expression. Adipokine circulating levels generally correlate positively with adiposity (133). Given eWAT mass and adipocyte size were similar in Restricted groups but increased compared to Control mice, we measured adipokine gene expression in all groups to determine whether expression paralleled fat mass. Despite no difference in adipose mass, gene expression of pro-inflammatory adipokines [interleukin-6 (IL-6), tumor necrosis factor α, (TNF-α)] and leptin in eWAT were significantly higher (2–6 fold) in Restricted (Gorging) mice compared to Restricted (Post-gorging) mice. Expression of markers of macrophage infiltration (F4/80, CD68) and plasma leptin were similarly higher. Although eWAT weight of Restricted (Post-gorging) mice was 31% higher than the Control group, gene expression of TNF-α and leptin in this group was significantly decreased (Figure 4.5B).
Figure 4.5 Gene expressions in eWAT in refed Restricted mice by metabolic state and relative to ad-libitum fed Controls. (A) Adipo/lipogenic and lipolytic mRNA expression. (B) Adipokines and inflammatory marker mRNA expression and plasma adipokines. Values represent means ± s.e.m., n=7–10 per group. Data were analyzed using one-wayNOVA with post hoc Tukey’s test. Different letters indicate statistically significant differences among groups, p < 0.05.
**Insulin Sensitivity.** To assess whether insulin sensitivity is altered in restriction-triggered gorging mice during refeeding, we used hyperinsulinemic euglycemic clamps to assess whole-body, hepatic, muscle, and adipose tissue insulin sensitivity. Eight Control mice (body weight: 18.3 ± 0.2 g) and seven Restricted mice (body weight: 19.0 ± 0.2 g) were successfully clamped on study days 14–17. Glucose infusion rates to maintain euglycemia (Figure 4.6A) during the experiment were approximately 1.5 fold lower in Restricted mice than in Control mice (Figure 4.6B) indicating whole-body insulin resistance. Clamp insulin levels were increased in Restricted mice (Figure 4.6C). Whole-body glucose uptake during the clamp was significantly decreased (24%) in Restricted mice (Figure 4.6D) although no significant differences in glucose disposal in gastrocnemius muscle or eWAT (Figure 4.6E) were detected. Basal endogenous glucose production (EGP) was 77% higher (Figure 4.6F) and the basal hepatic insulin resistance index was significantly increased (Control, 65.1 ± 13.8; Restricted, 194.2 ± 40.0, p < 0.05) in Restricted mice. Insulin-stimulated EGP was not different between groups.
Figure 4.6  Hyperinsulinemic-euglycemic clamp measurements of refed Restricted mice compared to ad-libitum fed Controls.  (A) Time course of arterial glucose.  (B) Glucose infusion rates during hyperinsulinemia.  (C) Basal and clamp insulin levels.  (D) Whole-body glucose uptake.  (E) Muscle and white adipose tissue glucose disposal.  (F) Endogenous glucose production (EGP) during basal and hyperinsulinemic conditions.  

Values represent means ± s.e.m., Control, n=7–8; Restricted, n=6–7, *p < 0.05.
4.5 Discussion

In the present study, gorging behavior was triggered in normal weight mice by restricting food over 5 days. When the mice were subsequently rationed food at levels approximating age-matched controls, the gorging persisted, and we compared anthropometric, metabolic, and gene expression measurements of the gorging mice in their two distinct metabolic states (Gorging and Post-gorging) to ad-libitum fed nibbling mice. We observed enhanced intra-abdominal fat accumulation despite controlled refeeding in restriction-triggered gorging mice which may be partially explained by suppressed BAT thermogenic capacity and a gene expression profile favoring lipid deposition. Additionally, we found robust intraday changes in adipose tissue gene expression and diminished insulin sensitivity in restriction-triggered gorging mice during weight regain.

Accelerated rates of fat recovery resulting in excess adiposity have been reported in adults and children during nutritional recovery after starvation (123) and in a rat model of controlled refeeding after semi-starvation (88-91). Moreover, during weight regain in women with anorexia nervosa, body fat was disproportionately redeposited centrally (134, 135). Consistent with these studies, refed Restricted mice gained significantly more intra-abdominal fat (eWAT) during weight regain despite a lower cumulative food intake and lower final body weight compared to ad-libitum fed mice. Analogous increases in adipocyte cross-sectional area substantiate this finding. Intra-abdominal adipose tissue mass and adipocyte size of refed Restricted mice, however, did not vary by
metabolic state. Thus, intraday changes in adipose tissue mass, if they exist, were not detectable as early as 3–4 h into gorging relative to mice that were 16–18 h post-gorging. We found no differences in subcutaneous fat mass after weight regain in refed Restricted mice compared to age-matched control mice, a finding also consistent with weight regain in women with anorexia nervosa (135).

*Increased* intra-abdominal adiposity in the face of *reduced* cumulative food intake would be possible with a sustained reduction in energy expenditure. Resting energy expenditure in Restricted mice would expectedly decrease with weight loss following food restriction. Additionally, food restriction has been shown to decrease energy expenditure beyond what would be expected from loss of body mass by suppressing thermogenesis – i.e. by increasing efficiency of energy utilization (95). Conversely, conditions such as overfeeding (e.g. gorging) have been shown to activate thermogenesis, including brown adipose tissue (BAT) thermogenesis (95, 136). Despite gorging meals, mRNA expression of UCP1, the mitochondrial protein determining thermogenic capacity in BAT, was significantly decreased in both Restricted groups compared to Control mice. This apparent sustained suppression of thermogenic capacity (hence energy expenditure) may partially explain the excess adiposity in Restricted mice after refeeding. In fact, in studies of catch-up growth (89) and weight regain following weight loss (94) in rats, spared energy from suppressed thermogenesis (increased metabolic efficiency) was shown to be preferentially directed to fat deposition.
In the present study we captured intraday changes in expression of lipid metabolism-related genes. That gene expression of key enzymes and transcription factors promoting *de novo* lipogenesis, adipogenesis, and lipid uptake in intra-abdominal adipose tissue were increased in Restricted mice during gorging was not surprising. However, at the end of the daily extended fast (Post-gorging), with the exception of SREBP-1, expression of lipogenic genes in Restricted mice remained elevated or were equivalent to fed controls suggesting fat storage is favored throughout the feeding cycle. This enhanced expression of lipogenic genes is consistent with a mouse model of catch-up growth following low birth weight (127) but varies slightly from a rat model of semistarvation-refeeding where both adipogenic and lipogenic gene expression were enhanced (91) although metabolic state was not reported in these studies.

In addition to intraday oscillations in adipo/lipogenic genes, adipokine gene expression also varied by metabolic state in restriction-triggered gorging mice, but did not parallel fat mass. In general, circulating levels of adipokines are positively related to fat mass (133), yet gene expression of pro-inflammatory adipokines and leptin during Restricted mice’s daily extended fast were negatively associated with fat mass relative to Control mice. This may best be explained by the fact that adipokine gene transcription is acutely regulated by nutrient availability and catecholamines. IL-6 and TNF-α mRNA expressions in adipose are positively regulated by glucose and glucosamine (137) while leptin transcription is negatively regulated by catecholamines and positively by nutrient availability (138). During Restricted mice’s daily extended fast, when plasma ketones
trended higher, nutrient availability presumably would be decreased and catecholamines increased. This would explain reduced IL-6, TNF-α, and leptin mRNA expressions as well as decreased plasma leptin levels. Altogether, these intraday variations in expression of lipid metabolism-related genes and adipokines demonstrate the exaggerated “fed” and “fasted” metabolic states consequent to gorging behavior.

A key study objective was to identify whole-body metabolic consequences of restriction-triggered gorging in mice during weight regain. Data from the hyperinsulinemic-euglycemic clamp experiments suggest hepatic and peripheral tissue insulin sensitivities in these mice are compromised. One explanation for decreased tissue response to insulin may lie with the duration of fasting. Studies in humans and rats have shown prolonged fasting induces peripheral and hepatic insulin resistance compared to shorter fasting durations or no fasting (101, 102, 139-141). In contrast, in C57BL6 mice, prolonged fasts (16–18 h) enhanced muscle insulin sensitivity (142, 143) and did not alter hepatic insulin sensitivity (143) compared to 4–5 h fasts. Our results resemble the studies in rats which demonstrated decreased whole-body glucose uptake (101, 102) and reduced hepatic insulin sensitivity(101) following prolonged fasting. Although the extended fast of Restricted mice in our study was shorter in duration than the prolonged fasts in the aforementioned studies, gorging mice in our study undergo this fast repeatedly (daily) over 14–17 days which could amplify the response especially relative to Control mice fasted once for the purpose of the clamp experiment.
Apart from the possible effects of fasting duration on insulin sensitivity, the initial food restriction imposed on Restricted mice may have also altered peripheral insulin sensitivity. In rats refed after semi-starvation to promote catch-up growth, glucose disposal in skeletal muscles was decreased while uptake in adipose tissue was increased (90). We measured glucose uptake in gastrocnemius muscle only and detected no difference between groups. However, because skeletal muscle is the major site of insulin-stimulated glucose disposal, our finding that whole-body glucose uptake is significantly decreased suggests glucose disposal in other skeletal muscles was diminished. Interestingly, the fold increase in glucose uptake in eWAT in our Restricted mice was similar to that observed in refed rats after semi-starvation. Thus, our refed Restricted mice may be similar to a rat model of catch-up growth where a state of hyperinsulinemia (a trend detected in our study) and muscle insulin resistance directs glucose to adipose tissue (90). In our mice, however, increased glucose uptake in adipose tissue did not completely compensate for decreased muscle disposal.

Finally, deleterious consequences of prolonged fasting and/or refeeding after food restriction offer potential explanations for diminished insulin sensitivities observed in our mice. During fasting, fatty acids released from adipose tissue for energy are stored in the liver for conversion to ketones. Therefore, a consequence of an extended fast is hepatic lipid accumulation which we observed in refed Restricted mice. Intra-hepatic lipid accumulation is associated with hepatic insulin resistance which is postulated to be mediated by lipid intermediaries (144). Similarly, a consequence of refeeding following
food restriction is enhanced accumulation of intra-abdominal fat (134, 135) – also observed in our mice – which itself is associated with hepatic and extrahepatic insulin resistance (145, 146). Thus, extended fasts and refeeding after food restriction both result in accumulation of lipids associated with increased risk of insulin resistance.

In summary, in the present study we show intra-abdominal fat accumulation is enhanced in mice during controlled refeeding following food restriction and suggest this may be partially explained by suppressed BAT thermogenic capacity and a gene expression profile favoring lipid deposition. We also demonstrate intraday oscillations in adipose tissue gene expressions in restriction-triggered gorging mice reflecting the exaggerated metabolic states consequent to gorging behavior. Importantly, we show diminished hepatic and peripheral insulin sensitivity are metabolic consequences of restriction-triggered gorging. Future studies should determine whether changes in adipose tissue gene expressions, lipid stores, and whole-body metabolism in our study can be attributed to the initial food restriction and/or gorging meal pattern.
4.6 Acknowledgements

This research was supported by the Carol S. Kennedy endowment, Ohio Agricultural Research and Development Center, Pelotonia Graduate Fellowship, and by grants from the National Institute of Health (R01 DK-40936, U24 DK-059635).
Chapter 5

Epilogue

Cancer cachexia is a metabolic disorder characterized by extensive weight loss during the later stages of the syndrome (25). Although reduced survival in cachectic patients is proximately attributed to skeletal muscle loss (25), a growing body of literature suggests that maintaining fat mass in cachexia may improve survival outcomes (8-10, 26). Few studies, however, have characterized catabolic processes contributing to adipose depletion in cachexia and none has investigated rates of lipolysis considering energy expenditure and adiposity. The aims of the first study were to characterize the catabolic events in adipose tissue in cachexia – that is, the catabolism of triglyceride stores (lipolysis) and processes promoting lipid utilization in adipose tissue. I focused on utilization of lipid within adipose tissue as it poses as an epicenter of energy wasting processes (e.g., adaptive thermogenesis or futile cycling) and consequently, avoidable lipid depletion. The mice were studied during the early stage of cachexia in an attempt to elucidate the mechanisms that initiate loss of adipose tissue.
The first study revealed that energy expenditure and markers of energy (lipid) utilizing processes (BAT thermogenesis) in adipose tissue were elevated in tumor-bearing mice in early cachexia with anorexia. Elevated energy expenditure was accompanied by an elevated lipolysis rate, presumably to meet energy demands as ectopic accumulation of lipid was not detected. What was most revealing about the findings, however, was that energy expenditure and markers of lipid utilizing processes in tumor-bearing mice were only elevated compared to pair-fed controls. This suggests that in early cachexia, energy utilizing processes may not be enhanced per se but rather these processes are not compensatorily suppressed in response to reduced food intake. Consistent with studies of food restricted mice (84, 94, 95), the saved energy was preferentially deposited as lipid in adipose tissue in pair-fed mice. Future studies should focus on the mechanisms by which the compensatory processes that spare lipid are overridden in tumor-bearing mice.

The strength of this first study was the utilization of regression analysis (ANCOVA) in analyzing metabolic data. In mouse studies, it is now recommended that energy expenditure data be analyzed using ANCOVA with relevant covariates (e.g., analyze both group and body composition effects) (147, 148). Dividing energy expenditure by body weight or lean body mass, a standard in the past, is suggested to result in erroneous estimates (148) and spurious interpretations (147) as metabolic rate is not linearly proportional to body weight. Future studies that adopt the recommended regression method of analysis will facilitate comparisons across studies. Similar recommendations have been made for measurements of lipolytic rate (118).
The final aim was to assess feeding behavior and characterize changes in adiposity in healthy mice subjected to a restricted feeding regimen. The impetus for this study was our lab’s observation of changes in feeding behavior and increased adiposity in pair-fed mice on restrictive pair-feeding regimens. This final study found that intra-abdominal fat mass was increased in restricted mice (despite reduced cumulative food intake) which was accompanied by elevated lipogenic and reduced uncoupling protein 1 gene expression. Although we did not measure energy expenditure, these results suggested that a compensatory suppression of thermogenesis (thus energy expenditure) and a gene expression profile favoring lipid deposition facilitated lipid accumulation in adipose tissue. The restricted mice were also observed to gorge their food allotments throughout the study which was reflected in the exaggerated intraday variations in expression of lipid metabolism-related genes and adipokines. This finding provides a cautionary tale regarding the importance of being cognizant of feeding behavior for the timing of metabolic measurements in mouse studies.

That wasting in adipose tissue in early cachexia may be the result of hypermetabolism and reduced food intake highlights the need for early nutritional intervention in cachexia and pharmaceutical drugs to suppress energy expenditure. Future studies to develop such therapies are warranted.
List of References


68. Souza SC, Palmer HJ, Kang YH, Yamamoto MT, Muliro KV, Paulson KE, et al. TNF-alpha induction of lipolysis is mediated through activation of the extracellular signal...


83. Hambly C, Simpson CA, McIntosh S, Dalglish GD, Speakman JR. Calorie-restricted mice that gorge show less ability to compensate for reduced energy intake. Physiol Behav. 2007 Dec 5;92(5):985-92.


thriftv metabolism: relevance for muscle-adipose glucose redistribution during catch-up


129. Matsuda M, DeFronzo RA. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care. 1999 Sep;22(9):1462-70.


van der Crabben SN, Allick G, Ackermans MT, Endert E, Romijn JA, Sauerwein HP. Prolonged fasting induces peripheral insulin resistance, which is not ameliorated by high-dose salicylate. J Clin Endocrinol Metab. 2008 Feb;93(2):638-41.


Miles JM, Jensen MD. Counterpoint: visceral adiposity is not causally related to insulin resistance. Diabetes Care. 2005 Sep;28(9):2326-8.


Appendix A: Lipolysis Assay

**Reagents** (adapted from (149))

**Incubation Solution (IS)**

- Krebs Ringer Bicarbonate HEPES Buffer
  - Krebs Ringer Buffer (Sigma K4002)
  - Sodium Bicarbonate 10 mM (Sigma S5761 - powder)
  - HEPES 30 mM (Sigma 3375 - powder)
    - Adjust pH to 7.4 with 5N NaOH

- Fatty Acid Free Bovine Serum Albumin Fraction V
  - 3% w/v (Calbiochem 126575)

- Isoproterenol
  - 10 μM (Calbiochem 420355)
    - Degrades 5% every 6 hours; make fresh before each experiment

**Glycerol Assay**

- Glycerol Standard (Sigma)
- Free Glycerol Reagent (Sigma F6428)
Tools & Supplies

Surgical Blades

Weigh Boats

5 mL polypropylene, 12 × 75-mm incubation tubes (VWR Scientific Cat # 60818-500)

2 ml microcentrifuge tubes

Equipment

Incubator

Thermometer

Solution Preparation (adapted from (149))

Incubation Solution (IS)

- Krebs Ringer Buffer (KRB) – mix powder in bottle with 1 L DI water
- For 10 mM Sodium bicarbonate (FW 84.01), add 0.841 g to KRB
- For 30 mM HEPES (FW 238.3), add 7.149 g to KRB

Incubation Media for Lipolysis Assay

Prepare fresh on the day of experiment

**Step 1.** Mix 1L IS as above. Check pH = 7.4. Adjust with 5N NaOH

**Step 2.** Mix IS + 3% albumin

Need 600 μL per sample

Example: For 60 samples, add 1.2 g albumin to 40 ml IS

**Step 3.** Stimulation Incubation Media (1x Isoproterenol = 10 μM)

Make 100x (1000 μM) isoproterenol:

112
- Add 12.4 mg isoproterenol HCL (FW 247.72) to 50 ml IS

Make 1x (10 μM) isoproterenol:

- For 20 ml (1x): (19.8 ml IS + albumin) + 200 ul (100x isoproterenol)

Step 4: Basal Incubation Media

20 ml of IS + albumin mix

**Lipolysis Assay** (adapted from (54))

Step 1: Adipose Tissue Excision

- Excise fresh fat pads from animals. Cut into 30 mg pieces (explants should not vary more than 2% by weight)

Step 2: Lipolysis Assay

- Place 30 mg explants in 600 ul assay buffer* in 12x75mm incubation tubes.
- Incubate for 3 h at 37°C (no shaking). After incubation, pipette 400 μL of infranatant into microcentrifuge tubes and store at −20°C until assayed for glycerol.

*Lipolysis Assay Buffers:

Stimulation Buffer: (IS + albumin) solution + 10 uM isoproteronol

Basal Buffer: (IS + albumin) solution

Step 3: Glycerol Assay

Materials: Glycerol Standard 26 mg/dl glycerol

Free Glycerol Reagent

Preparation: Reconstitute the Free Glycerol Reagent with 40 ml of DI water.
Stopper the vial, and immediately mix several times by inversion.

DO NOT SHAKE. Warm to room temperature.

Procedure:

- Make standards (based on optimization experiments)
- Pipette 10 ul standards and 20 ul samples into 96 well plate
- Add 200 ul glycerol reagent
- Incubate on rocker for 15 min
- Read at 540 nm on spectrophotometer
Appendix B: Lipolysis after 24 h Fast

Experiment

- 8 wk old male CD2F1 mice (n = 5) were fasted 24 h and then sacrificed
- Basal (unstimulated) and isoproterenol (10 μM) stimulated lipolysis rates were measured using 30 mg of excised epididymal white adipose tissue explants in incubation media as described in Appendix A. Results were compared to fed tumor free male CD2F1 mice (No Tumor group) from Chapter 3.

Results

- Unstimulated release of glycerol is reduced in 24 h fasted mice compared to fed controls (Figure B.1).

![Figure B.1](image)

Figure B.1  Basal and stimulated lipolysis in mice fasted 24 h. Different letters indicate statistically significant differences among groups, p < 0.05.
Appendix C: Adipocyte Analysis using ImageJ Software

**Purpose**: To calculate average perimeter, diameter, and area of adipocytes

Adapted from http://imagej.nih.gov/ij/docs/guide/

**Settings**

- **Set Scale**
  - File > Open - Open micrometer image
  - Use ‘Straight Line’ to draw a line a known distance on the micrometer
  - Analyze > Set Scale
    - Distance of Pixels will populate
    - Fill in ‘Known Distance’, ‘Unit of Length’, check Global and select OK. Note: for 200X magnification, ‘Distance of Pixels’ = 106 for ‘Known Distance’ of 50 μm.

- **Set Measurements**
  - Select Area, Perimeter, and Feret’s Diameter

**Analyze Particles**

- **Set Size** = (200 to 500) – 100000 (this captures small adipocytes, but avoids capturing artifacts)
- Set Show = Outline
- Select Display Data and Exclude Edges

**Procedure**

- File > Open – Open desired image (Uncheck Disable Global Calibrations)
  
  If Color, change to 16-bit

- Edit > Invert

- Image > Adjust > Brightness/Contrast
  - Use ‘Auto’ function and ‘Set’

- Image > Adjust > Threshold
  - Use ‘Manual Adjustment’ function and ‘Set’

- Process > Binary > Make Binary
  - Use default selections

- Process > Binary > Fill Holes

- Process > Binary > Watershed (not always appropriate)

- Use Freehand Selection to select desired area to evaluate

- Analyze > Analyze Particles

- Save Area, Perimeter, Diameter of each cell in spreadsheet

**Data Analysis**

- Calculate Area, Perimeter, Diameter for each group from ~150 adipocytes per mouse