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Cellular and Molecular Mechanisms Underlying Leptin's Metabolic Effects

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Cellular and Molecular Mechanisms Underlying Leptin's Metabolic Effects

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Dedication

This thesis is dedicated to my family and friends, whose support has been critical in anything I've managed to accomplish.

Acknowledgements

There are numerous people that I must acknowledge for contributions to this work and support in general. I would like to start by thanking Jeff Friedman for the privilege of working in his lab. In addition to being one of the most intelligent and discerning role models that I've been able to look up to, Jeff has been an incredible mentor, and more importantly a great friend. Working with Jeff has not only been a wonderful learning experience, it's been unbelievably fun. It's rare to find extremely successful scientists who are kind to their students and genuinely care about lab members, and for this I owe Jeff my deepest gratitude.

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collaborative project in his lab and for the invaluable mentoring he has provided. Nadeene Parker, Julie Buckingham, and Helen Boysen all worked with me in the Brand lab at Cambridge. John Strahler, who works in Philip Andrews' lab at Michigan, did the iTRAQ proteomic screen. Estella Gomez from the Schon lab at Columbia helped me establish a mitochondrial purification technique. Julie St. Pierre from the Spiegelman lab at Dana Farber provided my initial training in using the Clarke electrode. Tamas Horvath has provided great mentoring as well, and Zane Andrews from his lab worked with me for a few days. Sabrina Diano at Yale collaborated with us on the mitochondrial morphometry studies. Finally, the lab of Stan Hazen at the Cleveland Clinic looked at oxidated fatty acids with us to measure ROS.

Last but not least I would like to thank my family and friends for their support, guidance, and encouragement. I feel lucky to have parents that have showered me with unconditional love and made incredible sacrifices for me. My sister has been my best friend and has always kept me on the right track in many aspects of life. My little brother has always been supportive and I look forward to all the great things he's going to accomplish. Jonathan Kim has been a brother to me in many respects. Frankie O has been the best roommate anyone could ask for. There are countless other friends that are left unmentioned, Clint, Jas, Tarun, etc, etc. I look forward to having you all be a part of my life for years to come.

Table of Contents

List of Figures

List of Publications

Singh A, Wirtz M, Parker N, Hogan M, Strahler J, Michailidis G, Schmidt S, Diano S, Andrews P, Brand M, and Friedman J. Leptin-Mediated Changes in Liver, Skeletal Muscle, and Heart: a Look at Mitochondrial Metabolism, Structure, and Protein Levels. (In Preparation)

Abstract

Obesity is rapidly increasing in prevalence, and has become one of the leading contributors to poor health in the world. Increased body weight by necessity must be a result of increased nutrient intake relative to total energy expenditure. While many have focused on the importance of caloric consumption in determining weight, it is increasingly clear that differences in energy expenditure, basal metabolic rates and/or adaptive thermogenesis are also important variables that contribute to human obesity.

The experiments in this thesis start off by demonstrating that a significant portion of leptin-mediated weight loss is the result of an increase in energy expenditure caused by the hormone. They then go on to take a closer look at three candidate organs in which such changes in caloric expenditure are likely to be occurring. Our study finds that leptin treatment of *ob/ob* mice reduces the basal metabolic rate, uncoupled respiration (i.e. leak), and non-mitochondrial respiration of primary hepatocytes. We show that this is due to changes in the substrate oxidation system in liver mitochondria as well as changes in mitochondrial structure, and volume density. These changes are directly caused by leptin, and are not downstream of the hormone's correction of hepatic steatosis.

We next explored the proteomic and lipidomic modifications underlying the changes discussed above. Leptin-mediated changes in three components of the

mitochondrial respiratory chain can account for the aforementioned metabolic effects of the hormone in the liver. Dramatic alterations in levels of the enzyme ELOVL5 might be responsible for the hepatic steatosis exhibited with leptin deficiency, and for its correction with leptin treatment. Furthermore, differences in mitochondrial cardiolipin levels may also be associated with the observed leptin-mediated changes in the liver. Finally, we show a trend that leptin may be altering the levels of reactive oxygen species in hepatic mitochondria.

Having completed a thorough investigation into leptin-mediated changes in the liver, we moved on to the other two candidate organs, the heart and skeletal muscle. Our results show that leptin clearly modulates mitochondrial respiration in these tissues, though we cannot yet say exactly how it does so. In conclusion, this thesis illustrates the effects of leptin levels on the liver, and details the mechanisms by which the hormone induces change down to the proteomic level. It also shows clear leptin-mediated effects in skeletal muscle and the heart, and sets the stage for further investigation into the role of these tissues in leptin induced weight loss.

Chapter 1: Introduction

Obesity as a Global Health Problem

Defining Obesity and the Associated Physical and Psychological Stress

Obesity is loosely defined as an excess amount of body fat relative to lean body mass. The most commonly used method for categorizing individuals as being overweight or obese is body mass index (BMI), which is calculated by dividing one's weight in kilograms by the square of his/her height in meters. Waist circumference, skin fold thickness, and bioimpedance are other methods used in defining obesity. The World Health Organization defines individuals with BMIs over 25 as being overweight, and those with a BMI over 30 as obese (Committee, 1995; W.H.O., 1997).

Obese individuals are at a higher risk for developing coronary heart disease, type 2 diabetes mellitus, hypertension, cancer, strokes, sleep apnea, cholelithiasis, and osteoarthritis (Bennett, 1996; Kopelman, 2000). Studies have shown a positive correlation between mortality and body weight, with the lowest mortality being found in those who are slightly underweight (Lew, 1985). Both the prospective Nurses Health Study (Manson et al., 1995), which included approximately 115,000 women, and a 26-year follow-up of over 5,000 patients in the Framingham Heart Study (Hubert et al., 1983) showed an increased risk of mortality as BMI increases. Similarly, the American Cancer Society's Cancer Prevention Study found that a

higher BMI is associated with greater mortality (Stevens et al., 1998). Indeed, individuals with a BMI of 19 to 24.9 are at a 20% increased risk of death, which increases to 30% for a BMI over 25, 60% for a BMI over 27, and exceeds 110% for a BMI over 29 (Figure 1.1) (Calle et al., 1999).

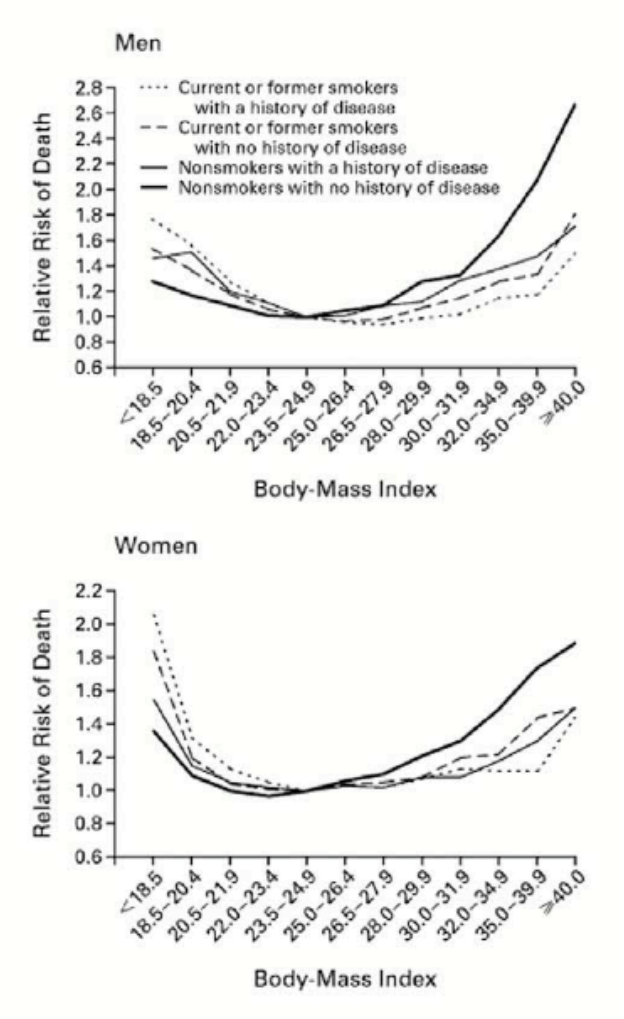


Figure 1.1 Body Mass Index vs. Relative Risk of Death. Increased BMI in both men and women is associated with a higher mortality risk. This risk rises exponentially with BMIs above 25-28. Figure exerpted from Calle et al. (1999).

In addition to incurring a higher risk of mortality, obese individuals are constantly subjected to social pressure as well as societal stigma, and thus face a tremendous pyscosocial burden (Friedman, 2003). Obese people often have a poor self image and impaired social skills, both of which are exacerbated by societal discrimination against the obese (Bennett, 1996; Crocker et al., 1993; Friedman, 2000). Overweight individuals are more likely to be categorized as having negative personality traits and thought of as being unhappy (Hiller, 1981). Furthermore,

studies have demonstrated that the obese are victims of prejudice and discriminatory behavior that negatively affects their education, employment, health care, and economic status (Bennett, 1996; Puhl and Brownell, 2001; Sarlio-Lahteenkorva et al., 1995). As a result of this stigmatization, obese individuals are subject to psychological distress, poor self-esteem, and sometimes worsening weight problems (Myers and Rosen, 1999).

Increasing Prevalence of Obesity

Obesity is rapidly emerging as a significant global health problem, and has joined the ranks of malnutrition and infectious disease as a leading contributor to poor health in the world (Kopelman, 2000). The most recent National Health and Nutrition Examination Survey (NHANES) indicates that 66% of adults in the US are either overweight or obese, a cohort that has been growing over the last fourty years (Ogden et al., 2006). Obese individuals made up 32.2% of the US adult population in 2004, as compared to 12.8% in 1962 (Kuczmarski et al., 1994). This trend manifests itself in younger individuals as well, and has shown an even more alarming rate of increase in children and adolescents. As a matter of fact, the prevalence of overweight children in the US has more than doubled since 1976 (Troiano et al., 1995; Yanovski and Yanovski, 2002). Since the presence of obesity in early infancy, childhood, and adolescence markedly increases the risk for obesity in adulthood, these youngsters are often predisposed to a lifetime of health problems (Dietz, 1994).

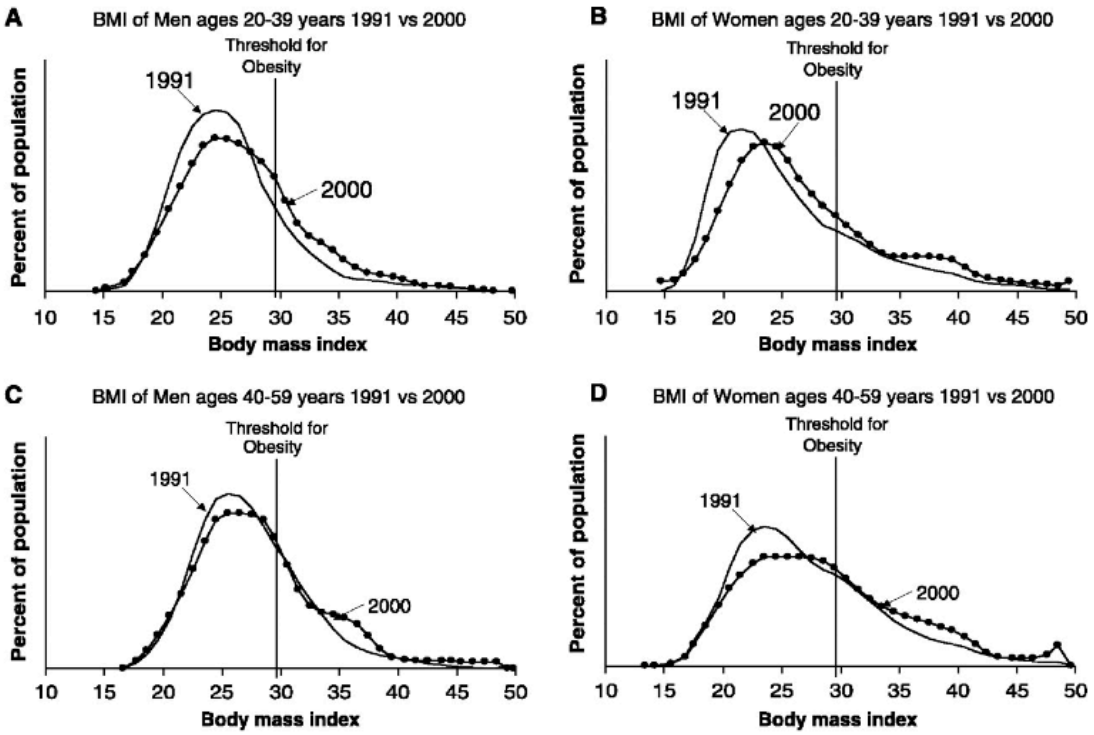


Figure 1.2 BMI distribution for men and women in the United States aged 20 to 39 (A and B) and 40 to 59 (C and D) for the years 1991 and 2000. In both of the age groups shown, the distributions have shifted rightward and become more skewed. Figure exerpted from Friedman (2003).

Though obesity is often viewed as a problem only encountered in countries of affluence, it is actually rapidly increasing in the developing world (Taubes, 1998). In fact, it is estimated that the number of obese people in the world is equivalent to those suffering from hunger (Campbell, 2000). Obesity has also taken a hold in countries such as Japan, Korea, China, and Thailand - nations where it has traditionally not been a concern (Popkin, 1994). Similar trends in obesity have been observed throughout Europe as well, though they are reported to be less severe (W.H.O., 1988). On the other hand, obesity rates amongst Pacific Islanders are astonishingly high, with a prevalence of over 60% in certain populations (Hodge et al., 1995; Shmulewitz et al., 2001).

When discussing trends in the prevalence of obesity, we must keep in mind that the disorder is defined as a threshold (i.e. BMI > 30), and thus relatively small increases in average weight can have a disproportionate effect on its incidence (Friedman, 2003). For example, while the incidence of obesity in the US increased by one third from 1991 to 2000 (i.e. 23.3% to 30.9%), the average weight of a typical American rose by approximately 7 to 10 pounds (Flegal and Troiano, 2000). Therefore, an increase in the average BMI of the US population from 26.7 to 28.1, lead to a marked increase in people with a BMI > 30, and hence the sharp rise in obese individuals (Figure 1.4) (Friedman, 2003).

Environmental Contributions to Obesity

Billions of dollars are generated in revenue every year by the diet industry, with over 30% of adults in the United States reporting that they are trying to loose weight (Friedman, 2000; Serdula et al., 1999). A fundamental concept presented in the marketing of many diets is the idea that adjustments to body weight are simply a matter of determination. The common oversimplification that obesity results solely from a lack of willpower, gluttony, and utter laziness has in recent years been challenged by a wealth of scientific knowledge. Clinical and molecular studies have shed light on the interplay between genes and the environment in leading to the disorder of energy imbalance, and it has become quite clear that both factors play a determining role when it comes to an individual's body weight.

The environment can be looked upon as contributing to the development of obesity in whichever ways it increases the frequency of behaviors that promote positive energy balance (Hill and Peters, 1998). In today's society, the availability of food, popularity of high calorie meals and drinks, and increased portion size, have all contributed to the increased prevalence of obesity. Even with the call to alarm against trends in weight gain, foods low in fats or carbohydrates, but high in calories, have been marketed as smart and guilt-free alternatives.

Alongside this increase in caloric intake over recent history, modernization has brought with it a decrease in physical activity. Studies have shown that children

spend an increased amount of leisure time watching television or playing video games, which may be contributing to the observed increase in childhood obesity (Robinson, 2001). This makes sense, as reduced physical activity and low energy expenditure are risk factors for weight gain (Ravussin et al., 1988a; Rissanen et al., 1991).

Since the human genome has not changed significantly in the last 20 years (Hill and Peters, 1998), much of the blame for the increase in obesity can be placed on environmental factors. This hypothesis is reinforced by studies of migrant populations, one of which shows that the likelihood of an Asian American individual being overweight or obese increases with the number of years he/she has spent in the US (Lauderdale and Rathouz, 2000). Another such study shows that obesity is more prevalent amongst American-born versus foreign-born individuals, and increases greatly in second and third generation immigrants (Popkin and Udry, 1998).

The Genetic Influence Over Obesity

In addition to the environmental contributions to body weight, there is a precise physiological mechanism rooted in genetics that exerts control over energy balance. Obesity genes code for the molecular components that regulate the homeostasis between energetic inputs and outputs, a system that precisely matches caloric consumption with caloric expenditure to maintain controlled stores of energy, mainly in the form of fat (Spiegelman and Flier, 2001; Zhang et al., 1994). Exemplifying this is the fact that a typical human being consumes approximately 10

million calories over the course of a decade, with generally only a modest change in body weight (Friedman, 2003). Calculations show that this trend requires food intake and energy expenditure to match one another within 0.17% (Weigle, 1994), a level of precision that exceeds the ability of nutritionists to count calories by several orders of magnitude. The 'set point' hypothesis buds from this inherent stability in body weight, and proposes that food intake and energy expenditure are coordinated to keep body weight relatively constant (Friedman and Leibel, 1992; Harris, 1990). In support of this theory, when a study restricted food intake to reduce body weight by 10%, it found that energy expenditure decreased to offset the attempted changes (Leibel et al., 1995).

If such a precise homeostatic mechanism for the regulation of body weight exists, then why has average body weight, and thus obesity, climbed so drastically over the last few decades? Shouldn't this physiological system resist changes in weight due to the abundance of calories or sedentary lifestyle that has become so pervasive in modern day society? To answer these questions, we can turn to the "thrifty gene hypothesis". This theory says that genes which allowed for the effective storage of energy as adipose tissue when food was scarce, as was the case in ancient hunter-gatherer societies, provided an adaptive advantage and were selected for over time (Neel, 1999). Thus, individuals that were able to store energy to use during times of food deprivation were able to survive longer and pass on the genes that allowed them to do so to a larger fraction of the next generation. The relatively sudden change

in environment that has made access to calorie dense food commonplace, has in the context of thousands of years of selection for "thrifty genes" made obesity a serious health problem.

If the change in environment has provided the bricks with which the house of obesity has been built, then genetic factors are the foundation holding it up. As much as 80% of the variance seen in BMI amongst individuals is attributable to differences in genetic makeup (Rosenbaum et al., 1997). An individual's genes govern his/her control over positive energy balance and defense system against developing obesity in a particular environment (Hill and Peters, 1998). Studies of adopted children, twins reared together or apart, and patterns of familial aggregation have all supported the importance of genetic factors in the determination of BMI. A study of over 100,000 people from either biological or adoptive relationships found a BMI correlation of 0.70 in monozygotic twins and 0.32 in dizygotic twins, which represents a heritability of 50-90% (Maes et al., 1997). This high heritability is equivalent to that of height and far exceeds that of many disorders generally considered to have a genetic basis (Allison et al., 1996).

Additional studies show that identical twins that have been reared apart have only a slightly lower correlation in BMI than those that have been raised together, and a shared childhood environment does not affect the similarity of their BMI later in life (Stunkard et al., 1990). On the flip side of the coin, adoptees raised together do not exhibit any correlation between BMIs (Stunkard et al., 1986). Furthermore, the rate

and proportion of weight gain, as well as the site of fat deposition, are all more closely linked in within pairs of identical twins than between them (Bouchard et al., 1990). This effect is also seen in mice, where genetically distinct inbred strains show very different amounts of weight gain in response to a high fat diet (West et al., 1992; West et al., 1994). A hypothesis that accounts for both the genetic foundation of obesity as well as the environmental contribution to it, proposes that susceptibility to obesity is under genetic control and that behavior and environment determine the phenotypic expression of inherited susceptibility genes (Barsh et al., 2000).

Body Energy Homeostasis

The Balance Between Energy Intake and Expenditure

Obesity, defined medically as a state of increased body weight to the extent that produces adverse health consequences, is a disease of energy imbalance. When energy intake exceeds energy expenditure, the excess calories are stored as fat. If this state of positive energy balance continues over time, so will weight gain that eventually leads to obesity. Assuming that an individual has no problems with the absorption of nutrients, energy intake will be directly proportional to the calories that he/she consumes.

Energy expenditure, on the other hand, is a bit more complex, and represents the conversion of oxygen and an energy source (fats, carbohydrates, and proteins) to carbon dioxide, water, heat and work on the environment (Lowell and Spiegelman, 2000). It takes the form of physical activity, basal metabolism, and adaptive

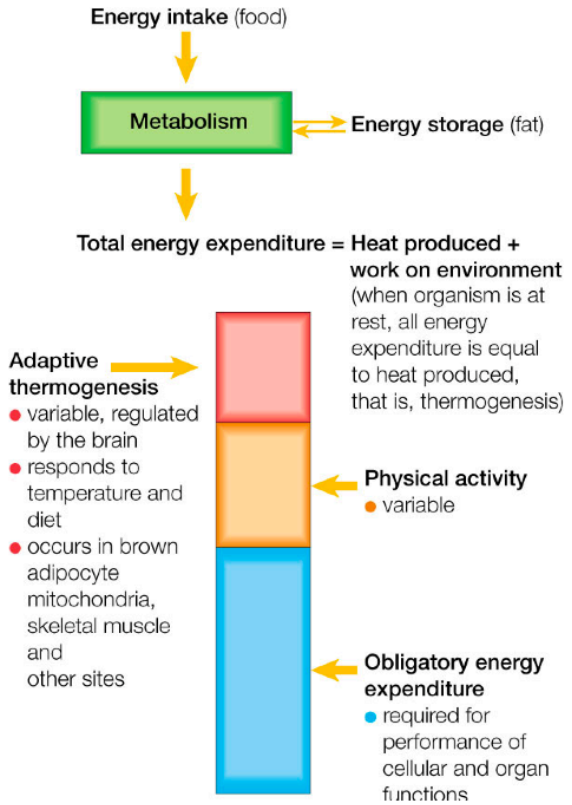


Figure 1.3 Thermodynamic Perspective of Energy Expenditure. Total energy expenditure can be subdivided into three principal components: obligatory energy expenditure, that resulting from physical activity, and adaptive thermogenesis. Figure excerpted from Lowell and Spiegelman (2000).

thermogenesis – which refers to energy dissipated in the form of heat in response to a change in the environment, such as cold exposure or diet alterations (Figure 1.3) (Lowell and Spiegelman, 2000). Basal metabolic rate (BMR) refers to the numerous biochemical processes required to sustain life, and is traditionally defined as the minimal caloric requirement for an organism to maintain life in the absence of external stimulation, work and growth (Harper et al., 2001a). There are many physiological processes that continue in an organism at this state, including blood circulation and respiration. The reactions that contribute to BMR on a cellular level include protein turnover, ion cycling across the plasma membrane, turnover of nucleic acids and lipids, and proton flux across the mitochondrial inner membrane (Brand, 1990; Hulbert and Else, 2000).

It is important to realize that a process can only cause or prevent obesity if it affects the overall energy equation of an organism (Spiegelman and Flier, 2001). An example of deviation from this understanding is the thought that limiting adipose tissue development can rectify obesity and its complications. However, experiments in which fat cell differentiation, development, and survival have been diminished show that this is not the case. Instead of producing healthy and obesity resistant organisms, these studies created mice that suffered from the lack of an appropriate depot in which to deposit excess energy, and exhibited a loss of critical adipocyte derived hormones (Ross et al., 1993; Shimomura et al., 1998). As a result, these mice had increased levels of blood lipids, fatty livers and diabetes, the combination of

which lead to death. Along these lines, when a genetically altered mouse is found to be leaner, one can conclude that an alteration has taken place in the total energy balance scheme – usually involving food intake or energy expenditure (Spiegelman and Flier, 2001).

Central Regulation of Energy Balance

The central nervous system (CNS) regulates energy balance, and thus body weight, through its effects on behavior (i.e. feeding and physical activity), the autonomic nervous system (regulation of energy expenditure and other aspects of metabolism), and the neuroendocrine system (i.e. growth hormone, thyroid hormone, cortisol, insulin, and sex steroids). The effectiveness of this system is highlighted by data showing that many species forced to gain weight by overfeeding, or lose weight by food restriction, will return to their control body weight levels upon return to voluntary food intake (Harris, 1990; Kandel et al., 2000).

The location of such a CNS energy regulation system was implicated through observations that animals with lesions in the hypothalamus demonstrated dysregulation of body weight and had excess fat mass (Harris, 1990; Hetherington and Ranson, 1942). Further studies showed that different regions of the hypothalamus have distinct contributions to energy regulation. Lesions of the lateral hypothalamus result in increased energy expenditure and decreased food intake, which work in concert to decrease body fat mass. Conversely, lesions in the ventromedial hypothalamus result in hyperphagia and obesity (Hetherington and Ranson, 1942;

Powley and Keesey, 1970). Thus, through these experiments it was hypothesized that the lateral hypothalamus was the feeding center of the brain, while the ventromedial hypothalamus was the satiety center. The identification of leptin as the signal which indicated lipid energy stores to these centers was crucial in uncovering how they maintain body weight homeostasis, and is discussed in the next section.

Short and Long Term Regulation of Energy Intake

The decision to begin and end feeding involves the integration of a number of factors, including food availability, metabolic status, hormone levels, and neural signaling – including visual, olfactory, emotional, and cognitive inputs (Schwartz et al., 2000b). The factors regulating food intake can be divided between short-term signals, which involve the initiation and termination of meals, and long-term signals, which promote regulation of caloric stores and energy homeostasis. Induction of satiety is the major determinant of meal size, and is the result of neural and endocrine inputs, such as gut distention and the release of cholecystokinin (CCK) (Moran, 2000). Regulation at the level of individual meal size is insufficient to account for energy homeostasis over longer periods of time, as mice repeatedly injected with CCK retain weight by ingesting a larger number of small meals (Spiegelman and Flier, 2001). Therefore, long-term signals that reflect the status of energy stores, such as the fat derived hormone leptin, are required to maintain energy balance.

A Circulating Signal of Energy Stores

Support for a circulating factor in the blood as the afferent signal of energy stores was popularized by the observation that transferring the blood of an obese animal with a lesion in the ventromedial hypothalamus into a lean animal caused the later to decrease food intake and lose body fat (Hervey, 1959). Kennedy had also proposed a "lipostatic theory", in which the hypothalamus responded to an adipose secreted "satiety factor" that circulated in the blood. He postulated that such a factor should have three defining characteristics: it should be produced by adipose tissue, it should circulate in the plasma, and it should be proportional to fat mass (Kennedy, 1953).

Further support for such a circulating signal of satiety came from Coleman's parabiosis experiments, in which the circulations of two mice were fused. In these studies, when a wild-type and *ob/ob* mouse were

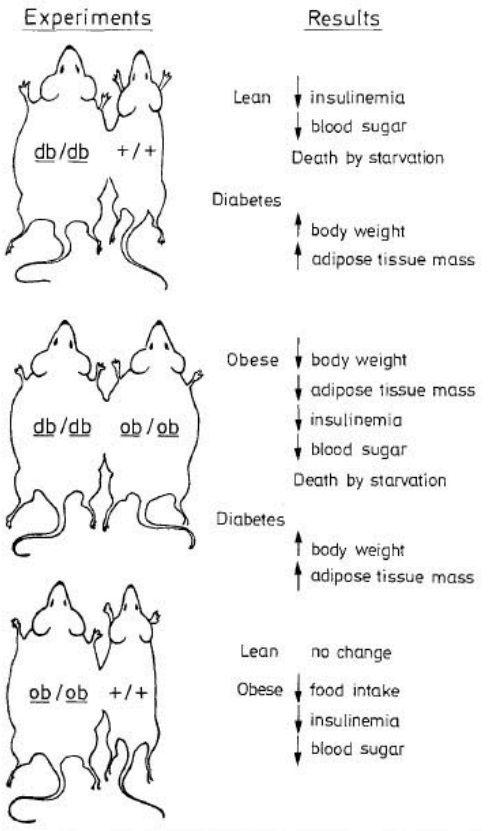


Figure 1.4 Coleman's Parabiosis Experiments. These studies suggested that the ob gene encoded a soluable factor and that the db gene encoded the receptor for this factor. Figure excerpted from Coleman, 1978.

fused, the *ob/ob* mouse decreased its food intake and lost weight, indicating that there is a circulating factor that the *ob/ob* mouse lacks (Figure 1.4, bottom panel). This factor is also present in the *db/db* mouse, which when fused with an *ob/ob* can make it loose weight as well (Figure 1.4, middle panel). The fusion of a *db/db* mouse with a wild-type mouse resulted in death of the wild-type mouse by self imposed starvation and no significant changes in the *db/db* animal, suggesting that the obesity seen in *db/db* mice may be the result of resistance to this factor (Figure 1.4, top panel).

Positional Cloning of the *ob* Gene, and Identification of Leptin

Kennedy's proposed "satiety factor" was discovered with the positional cloning of the ob gene, which was found to have high expressivity in adipose tissue (Zhang et al., 1994). The protein product of this gene was named leptin, from the Greek word *leptos*, meaning thin. Leptin fulfils all the requirements set by Kennedy for his factor: it is produced primarily by adipose tissue in nonpregnant animals (Zhang et al., 1994), it is a 16 kilodalton circulating member of the long-chain helical cytokine family (Zhang et al., 1997), and it is highly correlated with BMI in humans (Maffei et al., 1995).

Leptin conveys satiety by functioning as the afferent signal in a negative feedback loop regulating adipose tissue mass. When one is at his/her stable weight, the amount of leptin in circulation maintains body energy homeostasis by eliciting a state where food intake equals energy expenditure (Figure 1.5, top panel) (Friedman, 2000). A decrease in fat reserves reduces the amount of circulating leptin, which

signals to the hypothalamus to increase food intake and decrease energy expenditure (Figure 1.5, second panel). Conversely, increased fat reserves raise circulating levels of leptin, which triggers the hypothalamus to cut back food intake and increase energy expenditure (Figure 1.5, third panel). In most obese individuals there is a decreased sensitivity to leptin, and thus even at higher circulating levels of the hormone, they maintain body energy homeostasis by balancing food intake with energy expenditure (Figure 1.5, bottom panel).

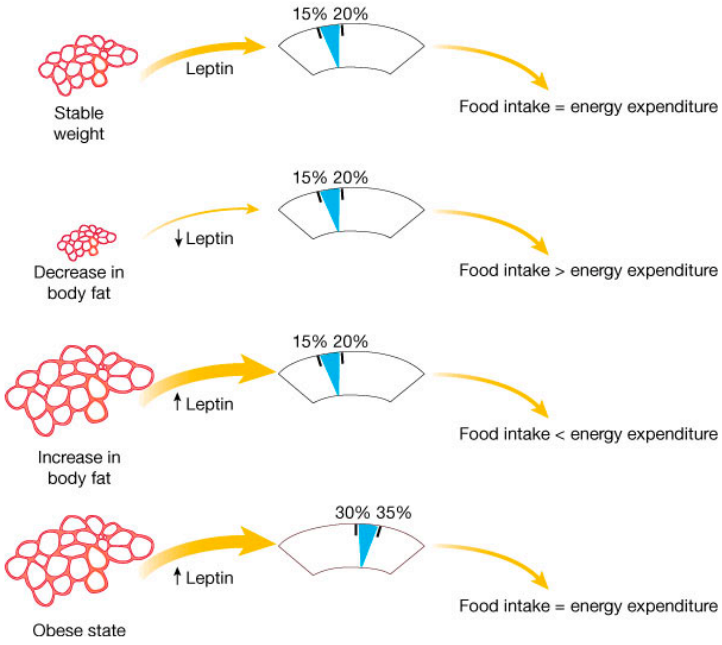


Figure 1.5 Leptin and the Regulation of Adipose Tissue Mass. Leptin is the afferent signal in a feedback loop regulating adipose tissue mass. Figure excerpted from Friedman (2000).

Mouse Models to Study the Function of Leptin

The *ob* knockout mouse model that lead to the identification of leptin can also be used to study the hormone. There are two such mutant strains of mice that are unable to produce leptin. The more commonly used ob^{1j} mutant has a nonsense mutation in the leptin gene, which results in a truncated protein that is not secreted into circulation (Zhang et al., 1994). The levels of *ob* RNA are elevated in the adipocytes of this mutant, suggesting that leptin expression may be under feedback

regulation. In the ob^{2j} mutant, on the other hand, the first intron of the gene has an Etn transposon inserted into it. This generates hybrid RNAs where the splice donor of the non-coding first exon is joined with the splice acceptor of the transposon, which prevents the formation of mature RNA (Moon and Friedman, 1997).

The defect in the production of leptin in these *ob* mice leads to animals that are hyperphagic and hypometabolic, rendering them obese from a very young age. In addition to being obese, *ob/ob* animals are diabetic, hypothermic, sterile, and show decreased immune function (Coleman, 1978). These mice behave very much like animals that have been starved, since in the absence of leptin their bodies are under the perception of being in extreme negative energy balance. Thus the functions less critical for short-term survival in a nutritionally deficient state, such as reproductive capability, are forgone to conserve as much energy as possible.

Restoration of physiological levels of leptin by intraperitoneal injection or subcutaneous infusion of the hormone in *ob/ob* mice leads to a dose-dependent decrease in body weight and food intake, corrects the metabolic defects discussed above, and restores fertility (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Furthermore, leptin treatment of these mice improves their hyperglycemia, hyperinsulinemia, and hypercorticosteronemia (Pelleymounter et al., 1995; Stephens et al., 1995). It is interesting to note that the reduction in body weight resulting from leptin treatment is specifically due to the depletion of adipose tissue and does not involve any changes in lean body mass (Halaas et al., 1995). In

support of this observation, adenovirus-mediated leptin gene therapy specifically depletes fat mass as well (Chen et al., 1996a).

The Leptin Receptor

Another monogenic mutation inherited in an autosomal recessive fashion, which also left animals obese and diabetic, was described alongside *ob* (Coleman, 1978). Mice with this mutation were called *db*, for diabetic, and the protein product of this gene was later shown to code for the leptin receptor Ob-R (Chen et al., 1996b; Tartaglia et al., 1995). Ob-R is a receptor that spans the plasma membrane, and has five alternatively spliced variants (Chen et al., 1996b; Chua et al., 1996; Lee et al., 1996). The C57Bl/Ks strain of db mice has an insert that leads to an alternatively spliced transcript containing a premature stop codon, which inhibits the expression of Ob-Rb. Other isoforms of the Ob receptor, however, are not affected by this mutation and remain intact (Chen et al., 1996b; Lee et al., 1996). The fact that C57Bl/Ks mice exhibit the same phenotype as animals unable to make any of the Ob-R isoforms suggests that Ob-Rb is the receptor splice variant that is critical in detecting leptin levels. In other studies, leptin had no effect on STAT or JAK activation in cells that only expressed Ob-Ra, providing further support for the importance of Ob-Rb (Ghilardi and Skoda, 1997; Ghilardi et al., 1996).

This long form of the ob receptor, Ob-Rb, is the only splice variant with an intracellular domain capable of transducing signals. When leptin binds to this receptor, the later is phosphorylated on at least two intracellular tyrosine residues,

namely Tyr985 and Tyr1138 (Li and Friedman, 1999). The phosphorylation of Tyr1138 is followed by the phosphorylation of JAK-2 and STAT-3, which leads to suppressor of cytokine signaling (SOCS)-3 transcription (Banks et al., 2000). Along these lines, cells transfected with the mutant receptor found in *db/db* mice showed defective STAT signaling (Ghilardi et al., 1996). Furthermore, leptin injection leads to a dose-dependent activation of STAT-3 in the hypothalamus of wild-type and *ob/ob* mice, but not *db/db* mice (Vaisse et al., 1996). Finally, leptin induces the expression of the STAT-3 target gene *fos* in the hypothalamus (Woods and Stock, 1996).

Once SOCS-3 transcription has been activated, Tyr985 comes into play, as it is needed in the negative regulation of the leptin receptor by SOCS-3 (Bjorbak et al., 2000). Peripheral administration of leptin has been shown to up-regulate hypothalamic levels of SOCS-3 mRNA (Bjorbaek et al., 1998). SOCS proteins negatively feedback on receptor signaling in response to cytokine stimulation, and levels of SOCS-3 remain up regulated for 20 hours after leptin administration, indicating long-term effects. Furthermore, SOCS-3 is over expressed in the hypothalamus of A^y obese mice, and its over expression in cell culture leads to inhibition of Ob-Rb signaling. These observations suggest that SOCS-3 may be involved in leptin's role in resisting obesity (Bjorbaek et al., 1999; Bjorbaek et al., 1998).

In addition to playing a role in the negative regulation of the leptin receptor, Tyr985 leads to docking and phosphorylation of the SH2 domain containing protein tyrosine phosphatase (SHP)-2 (Carpenter et al., 1998; Li and Friedman, 1999). SHP-2 decreases Jak2 phosphorylation, and thus blunts leptin signal transduction (Carpenter et al., 1998; Li and Friedman, 1999). Protein-tyrosine phosphatase, PTP1B, also decreases Jak2 phosphorylation, and thus mice lacking the former have increased levels of phosphorylated STAT-3, and are resistant to obesity (Cheng et al., 2002; Cook and Unger, 2002; Elchebly et al., 1999; Zabolothy et al., 2002). The full significance of these signaling cascades remains to be demonstrated in *vivo*, and a deeper understanding of them may be valuable in comprehending physiological disturbances resulting in obesity.

Ob-Rb has been detected in T cells, lymph nodes, the jejunum and vascular endothelium, however its highest expression seems to be in hypothalamic neurons (Chen et al., 1996b; Ghilardi et al., 1996; Lord, 1998; Morton et al., 1998). In situ hybridization studies have shown the receptor to be located in the arcuate nucleus, paraventricular nucleus (PVN), dorsomedial hypothalamic nucleus (DMH), ventromedial hypothalamic nucleus (VMH), and lateral hypothalamus (LH) – brain regions known to be involved in regulating appetite and body weight (Elmquist et al., 1997; Elmquist et al., 1998; Fei et al., 1997; Hetherington and Ranson, 1942; Mercer et al., 1996).

Currently, a definitive role for the other isoforms of Ob-R remains to be established. Ob-Ra and Ob-Rc are expressed on the microvasculature of the brain, and it has been suggested that they may be involved in leptin transport across the blood brain barrier (Banks et al., 1996; Hileman et al., 2002). Another splice variant, Ob-Re, is a soluble isform of the receptor, which appears to bind leptin in the plasma and assist in its action (Huang et al., 2001; Li et al., 1998).

Leptin's Role in Body Weight Regulation

Central vs. Peripheral Mechanisms of Action

The hypothalamus is the master regulator of energy balance in the brain, and is a major center of integration for leptin signaling. Ob-Rb is highly expressed in the hypothalamus and rats with lesions in this brain region exhibit leptin resistance, indicating that leptin has direct effects on the brain (Satoh et al., 1997). Intra-cerebro-ventricular (I.C.V) injection of leptin reduces food intake, increases energy expenditure, and results in weight loss (Campfield et al., 1995). Furthermore, leptin administered at extremely low doses by this means elicits the same weight loss and anorectic effects as much higher doses given peripherally (Halaas et al., 1997; Stephens et al., 1995). Administered peripherally, a leptin dose of 200ng/hr in *ob/ob* mice and 500ng/hr in wt mice is required for complete depletion of adipose tissue, and even higher levels are required when the hormone is given as twice daily injections (Halaas et al., 1995). On the contrary, chronic I.C.V. injection of just 3 ng/hr produces the same effects without increasing circulating leptin levels (Halaas et al., 1997). Furthermore, leptin rapidly induces STAT-3 DNA binding in the hypothalamus in a dose dependant manner, an effect that is not seen in any other tested tissues (Vaisse et al., 1996). Leptin administered either peripherally or centrally does not induce STAT-3 or cause weight loss in db/db mice (Halaas et al., 1995; Vaisse et al., 1996). Finally, brain specific expression of leptin receptors in receptor deficient mice can reverse many aspects of leptin deficiency (Kowalski et al., 2001).

As a whole, these data strongly suggest that leptin's actions are mediated in a large part through the hypothalamus.

It is important to keep in mind, however, that the leptin receptor is broadly expressed and leptin does have some direct effects on peripheral tissues. For example, leptin can influence the immune system, angiogenesis, and bone metabolism (Ducy et al., 2000; Lord, 1998; Sierra-Honigmann et al., 1998). More specifically, leptin has been reported to have effects on immune cells, muscle, liver, fat, heart, pancreatic β-cells, and other cell types (Kim et al., 2000; Lord, 1998; Minokoshi et al., 2002; O'Rourke et al., 2001; Shimabukuro et al., 1997). Interestingly, both neural pathways and direct actions in the periphery mediate leptin's activation of AMP kinase in peripheral tissues, which plays a role in regulating the oxidation of lipids (Minokoshi et al., 2002). That being said, the weight reducing effects of leptin which are the main focus of this thesis, are in large part mediated by the hypothalamus as discussed above.

The Leptin Regulated Melanocortin Pathway

Targeted deletions and positional cloning of genes, functional neuroanatomy, and identification of mutant genes in human obesity have all made it possible to gain insight into the complex central circuits that regulate energy balance. Though there are a number of brain centers that express leptin receptors and respond to the hormone, the best characterized and most clinically relevant of these help comprise the leptin-regulated melanocortin circuit. Seven unique proteins have been shown to

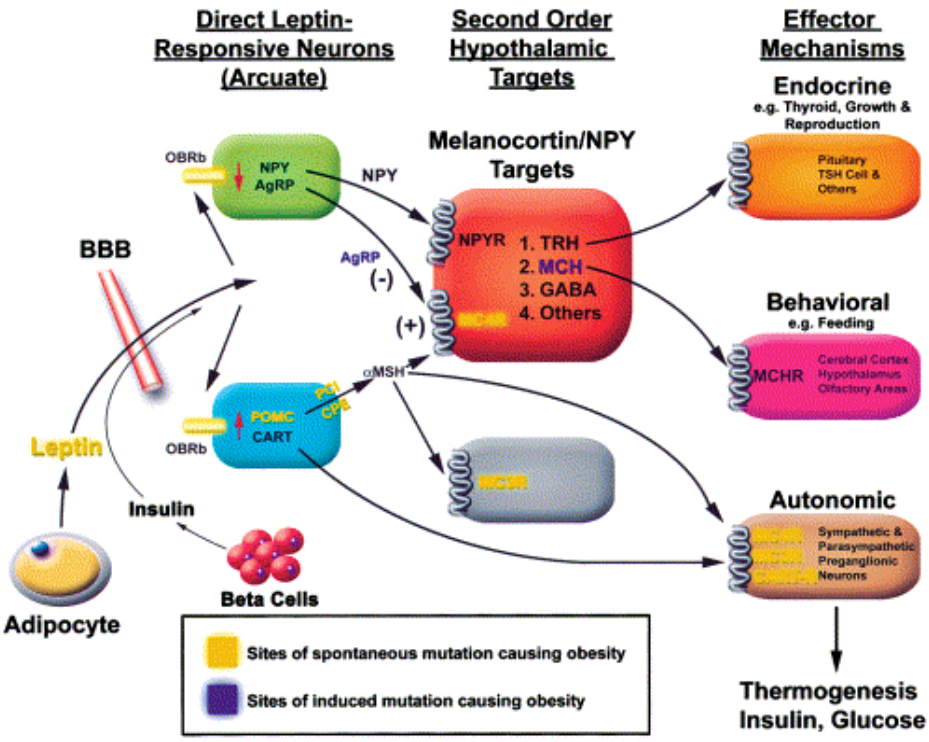


Figure 1.6 The Leptin-Regulated Melanocortin Circuit. The hypothalamic leptin-regulated melanocortin circuit controls energy homeostasis and body weight. Figure excerpted from Spiegelman and Flier (2001).

contribute to weight regulation and obesity in the major components of this circuit, which are detailed in Figure 1.6 (Spiegelman and Flier, 2001). In this circuit, leptin acts on two distinct populations of neurons in the arcuate nucleus, namely NPY/AgRP cells and POMC/CART cells. NPY and AgRP are orexigenic neuropeptides, and leptin action reduces their expression (Elias et al., 1999; Elmquist et al., 1999b; Schwartz et al., 2000b). POMC and CART, on the other hand, are anorexigenic neuropeptides, and leptin action induces their expression (Elias et al., 1999; Elmquist et al., 1999b; Schwartz et al., 2000b). Therefore, leptin signaling in the arcuate nucleus through Ob-Rb receptors suppresses two orexigenic peptides and induces two anorexigenic peptides.

These neuropeptides then go on to influence second order targets in the hypothalamus. POMC neurons produce αMSH, which stimulates MC4R receptors found in the brain and reduces food intake. On the other hand, AgRP works to inhibit MC4R receptors, which increases feeding and diminishes the hypophagic response to leptin (Cone, 1999; Fan et al., 1997). Transgenic and pharmacologic experiments have shown that the dominant obesity syndrome exhibited by the A^y mouse is due to ectopic expression of the agouti protein that produces obesity by antagonizing the action of αMSH on MCR4 receptors in the brain (Fan et al., 1997; Graham et al., 1997; Lu et al., 1994; Ollmann et al., 1997). The action of agouti in these mice mimics that of AgRP, which is its hypothalamic homolog.

Mice with one copy of the MC4R gene knocked out display moderate obesity, and those with a homozygous deletion of the receptor are fully obese (Huszar et al., 1997). In humans, approximate 4-5% of severe obesity is due to mutation at this location. The disorder in these individuals seems to arise due to haploinsufficiency rather than a dominant negative mechanism (Farooqi et al., 2000). These findings highlight the importance of the melanocortin pathway in maintaining normal energy balance, and suggest that it is extremely tightly regulated (Spiegelman and Flier, 2001).

The melanocortin pathway is also involved in a number of other human and murine causes of obesity. One such example, which leads to obesity in both organisms, occurs due to a mutation in the POMC gene that prevents the synthesis of

αMSH (Krude et al., 1998). Other such mutations that function through POMC processing occur in the enzymes PC-1 and CPE, and cause obesity in humans and mice respectively (Barsh et al., 2000). Obesity in mice also results from the targeted deletion of the MC3R gene, which codes for receptors closely related to MC4R (Butler et al., 2000). Since the obesity observed with MC3R deletions occurs without the hyperphagia seen in deletions of MC4R, it is thought that they are involved in distinct physiological pathways. Melanocortin systems, along with serotonin pathways, may also be involved in cachexia and eating disorders such as anorexia nervosa (Marks et al., 2001).

Leptin and the Melanin Concentrating Hormone Circuit

Lesion studies have implicated the lateral hypothalamus and zona incerta as "feeding centers" of the brain. Melanin concentrating hormone (MCH), another hypothalamic peptide involved in weight regulation, is exclusively expressed in large neurons whose cell

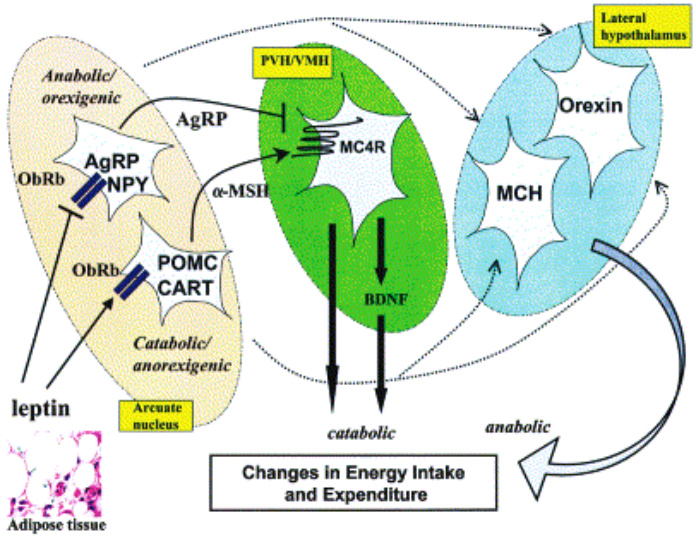


Figure 1.7 Leptin-Regulated Hypothalamic Circuits. Recent studies suggest that neurons in the lateral hypothalamus expressing MCH may be involved in leptin-mediated control of energy balance. Figure excerpted from Flier (2004).

bodies reside in these areas (Flier, 2004a)(Figure 1.7). Research has also confirmed a connection between arcuate neurons expressing AGRP/NPY and POMC/CART, and neurons in the lateral hypothalamus expressing MCH (Elias et al., 1999). Over-expression of MCH in the brain results in obesity and increased susceptibility to a high fat diet (Ludwig, 2002). Conversely, deletion of the MCH gene produces a mouse that is lean as the result of hypophagia and increased energy expenditure (Shimada et al., 1998). Knocking out this gene also substantially suppresses many aspects of the *ob/ob* phenotype (Segal-Lieberman et al., 2003).

The MCH receptor, MCHR1, is a G protein coupled receptor that is expressed widely in the brain. Targeted deletion of this receptor produces a mouse that is lean due to increased energy expenditure rather than reduced caloric intake (Marsh et al., 2002). Antagonists to the MCHR1 receptor suppress food intake and resist the obesity that develops in mice fed a palatable diet (Borowsky et al., 2002).

Obesity, Energy Expenditure, and the Role of Leptin

Energy Expenditure is Defective in Obesity

There are many lines of support for defects in energy expenditure underlying obesity, the strongest of which come from monogenic rodent models such as *ob/ob*, *db/db*, and melanocortin-4 receptor gene knock out mice (Lowell and Bachman, 2003). In all three of these genetic models, when food intake is restricted to that of wild-type controls, marked obesity still develops (Coleman, 1978). Furthermore, animals in which hypothalamic lesions are induced also become obese when food restricted to sham-treated control levels (Bray and York, 1979). Indeed, there are a few exceptions to the rule that animal models of obesity, whether generated through genetic manipulation or induced through lesions, have decreased energy expenditure (Lowell and Bachman, 2003). Studies also suggest that obesity in humans is caused at least in part by reduced energy expenditure. For example, low energy expenditure, normalized for lean body mass, has been shown to be predictive of future weight gain (Ravussin et al., 1988a). Thus comprehending the role of energy expenditure in obesity is critical to understanding the disorder and devising therapies to combat it.

Standard Metabolic Rate

As discussed earlier, basal metabolic rate (BMR) represents the minimal calories required for an organism to maintain the basic functions of life in the absence of external stimulation, work and growth. BMR and resting metabolic rate (RMR) are difficult and not suitable to measure in animals, and thus standard metabolic rate

(SMR) is used instead (Harper et al., 2001b). SMR is defined as the amount energy expenditure for an adult organism that is awake but resting, not digesting food, and is at thermoneutrality – the environmental temperature at which no energy has to be expended to maintain body temperature (Lowell and Spiegelman, 2000). There is a large spectrum of varying SMRs across phyla, and endotherms have a five fold greater metabolic rate than ectotherms of the same size (Rolfe and Brown, 1997). Furthermore, SMR within a phylum is inversely correlated with body size (Porter and Brand, 1993). The differences in SMR that exist amongst phyla and species suggest the presence of regulatory mechanisms that may be manipulated pharmacologically to change energy balance and increase adaptive thermogenesis (Harper et al., 2001b).

Factors Affecting Adaptive Thermogenesis

Standard metabolic rate and other such measurements represent the conversion of an energy source (food or stored) and oxygen to carbon dioxide, water, heat and work on the environment. The presence of heat in the equation above highlights the fact that many reactions in energy metabolism, such as those that consume ATP or are catalyzed by the mitochondrial respiratory chain, are exothermic in nature. Adaptive thermogenesis is defined as heat production in response to environmental temperature or diet (Lowell and Spiegelman, 2000). This component of energy expenditure serves to protect an organism from cold exposure or changes in energy homeostasis after modifications in diet.

The environmental temperature can greatly influence energy expenditure in an organism, as highlighted by the fact that oxygen consumption increases by approximately 2-4 fold in rodents after both acute and chronic incubation at 4^{o}C (Davis et al., 1960; Depocas et al., 1956). Shivering plays a role in maintaining body heat in the initial response to this temperature, however with adaptation it disappears and other mechanisms, including adaptive thermogenesis, take over (Davis et al., 1960; Foster and Frydman, 1979). Though the effect seen in humans is smaller, changes in environmental temperature show a similar influence over adaptive thermogenesis. For example in identically clothed humans, a reduction in ambient temperature from 28^{o}C to 22^{o}C causes a 7% increase in heat production (Dauncey, 1981).

Food intake also wields influence over adaptive thermogenesis. For example, starvation can decrease resting metabolic rate by up to 40% (Blaxter, 1989). Along these lines, adhering to a diet that is able to maintain a 10% reduction in body weight is associated with decreased energy expenditure (Leibel et al., 1995). The value of such a regulatory mechanism is clear for times in which the availability of caloric sources may be scarce, however it serves to counter voluntary weight loss as a result of dieting. In the face of this reduced energy expenditure, one must maintain a disproportionately low caloric intake to maintain weight loss – a feat that makes successful dieting incredibly difficult and leads to the failure of most diets over time (Friedman, 2003).

Feeding has the opposite effect on adaptive thermogenesis, and in both rodents and humans can increase energy expenditure acutely and in the long term. Acutely, feeding can raise metabolic rate by 25-40%, which is referred to as the thermic effect of food (Shibata and Bukowiecki, 1987; Sims and Danforth, 1987). Increased caloric intake over a prolonged period of time can also increase energy expenditure, which protects against the development of obesity (Levine et al., 1999). Interestingly, this adaptive response to overfeeding is influenced by genetics (Bouchard et al., 1990), and varying genetic backgrounds could leave individuals differently prone to developing obesity.

Central Control of Adaptive Thermogenesis and the Role of Leptin

As was mentioned earlier in our discussion of the hypothalamic influence over energy homoeostasis, destruction of neurons in the hypothalamus results in obesity (Elmquist et al., 1999b). The obesity in these cases is usually associated with increased caloric intake, however even if food is restricted to the level of control animals, obesity still develops (Himms-Hagen, 1989). Furthermore, mutations in leptin and its receptor, MCR4, and MCH all influence both food intake and energy expenditure in a concerted way to influence fat storage (Spiegelman and Flier, 2001). These findings, along with others, suggest that the hypothalamus has the capacity to control adaptive thermogenesis (Lowell and Spiegelman, 2000).

There are several lines of evidence suggesting that the sympathetic nervous system is the main efferent pathway through which the hypothalamus exerts its

control over adaptive thermogenesis (Lowell and Bachman, 2003). First of all, cold exposure and diet increase sympathetic nerve activity (Landsberg et al., 1984). Secondly, exogenous administration of epinephrine and norepinephrine, both *in vivo* and *in vitro*, stimulates energy expenditure. Thirdly, brown adipose tissue is highly innervated by sympathetic nerves, and the thermogenic activity of it is completely dependent on intact sympathetic stimulation (Himms-Hagen, 1989). Our current understanding of diet-induced thermogenesis is summarized in figure 1.8 (Lowell and Bachman, 2003). As shown in the figure, diet and leptin act on the hypothalamus and regulate sympathetic nerve activity to increase energy expenditure in different tissues.

While much focus has been directed on the importance of leptin's role in the

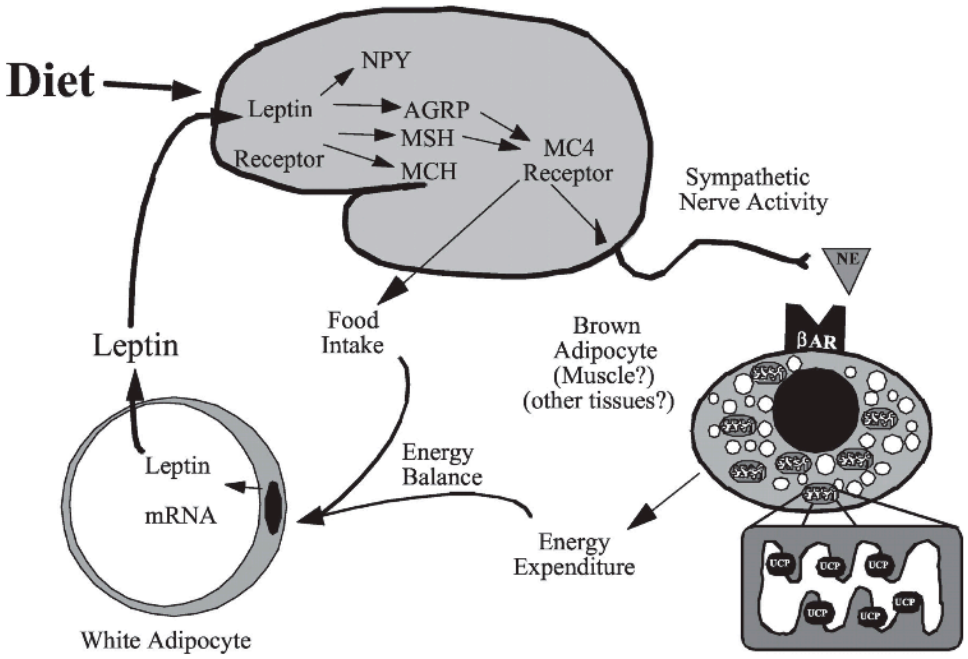


Figure 1.8 The Pathway Responsible for Diet-Induced Thermogenesis. Diet and leptin act on the hypothalamus and regulate sympathetic nerve activity to increase energy expenditure in different tissues. Figure excerpted from Lowell and Bachman (2003).

control of food intake, it is increasingly clear that differences in energy expenditure, basal metabolic rates and/or adaptive thermogenesis, are also important variables that contribute to human obesity (Bouchard et al., 1990; Levine et al., 1999; Ravussin, 1995). This is because in addition to reducing caloric intake, leptin infusion of *ob/ob* mice increases total energy expenditure, selectively promotes fat metabolism, and prevents the fall in adaptive thermogenesis that normally occurs with reduced caloric intake (Doring et al., 1998; Halaas et al., 1997; Hwa et al., 1997; Kamohara et al., 1997; Mistry et al., 1997; Pelleymounter et al., 1995; Scarpace et al., 1997; van Dijk et al., 1999). By adjusting for changes in physical activity, it has been shown that leptin infusion doubles the amount of feeding related adaptive thermogenesis by acting on both the amplitude and duration of it, and decreases postprandial RQ (Ruffin and Nicolaidis, 2000). This conclusion is consistent with the finding that stimulation of the ventromedial hypothalamus (VMH), a brain center through which leptin exerts some of its catabolic effects as discussed earlier, increases metabolism and decreases RQ (Ruffin and Nicolaidis, 1999), while lesions in this area cause obesity (Elmquist et al., 1999b). Therefore, a significant portion of leptin-mediated weight loss can be attributed to an increase in energy expenditure, including adaptive thermogenesis, through the pathway outlined in figure 1.8.

Target Tissues Mediating Leptin Driven Adaptive Thermogenesis

While the activation of adaptive thermogenesis in brown adipose tissue by sympathetic nerve signaling via β-adrenergic receptors and cyclic AMP is well

established, the induction of skeletal muscle and other tissues by this mechanism remains to be studied in more detail (Lowell and Spiegelman, 2000). Support for similar control over these tissues comes from observations that catecholamine infusion into skeletal muscle increases energy expenditure without the performance of work (Simonsen et al., 1992). Furthermore, knockout mice unable to produce catecholamines show greater sensitivity to cold than do mice mutated in UCP-1 (Enerback et al., 1997; Thomas and Palmiter, 1997). These studies suggest that pathways outside of brown adipose tissue are important contributors to adaptive thermogenesis.

While leptin's effects on energy expenditure can be easily demonstrated at the level of the whole organism, the tissues responsible for this effect as well as the underlying mechanisms are unknown (Lowell and Spiegelman, 2000). Possibilities for such tissues include skeletal muscle, liver, white adipose tissue and the heart, and are sites of interest as they could be relevant to body weight control in humans (Lowell and Bachman, 2003). Several studies have suggested that the liver may mediate leptin's effects on metabolism. This possibility has been suggested based on the liver's integral role in lipid metabolism, its significant energetic demands of ~20% of standard metabolic rate (SMR) in the rat (Porter and Brand, 1993), and its contribution to the catabolic effects of leptin (Cohen et al., 2002). Skeletal muscle and the heart are other highly metabolic organs (Rolfe and Brand, 1996; Rolfe et al., 1999), which have also been suggested to play a role in the increased energy

expenditure associated with leptin treatment. However leptin's effects on metabolism or mitochondrial function in these tissues has not been directly studied.

Chapter 2: Materials and Methods

Leptin Administration

Male C57BL6/J and C57BL6/J *ob/ob* mice (8-10 weeks old) were purchased from The Jackson Laboratory (Barr Harbor, ME), and housed in individual cages in a 22^{o}C temperature controlled room, or 30^{o}C incubator (for thermoneutral studies). Alzet miniosmotic pumps with a flow rate of 0.5 µl/hr were filled aseptically with either sterile PBS or Leptin (400 ng/µl for *ob/ob* mice ; 1 µg/µl for wt mice ; 5µg/µl for wt NC and wt FF mice). The pumps were incubated in a sterile 0.9% NaCl solution for at least six hours, and implanted s.c. using isoflurane as an anesthetic. For the withdrawal studies, all pumps were surgically removed from treated and control animals after eight days, and mice were sacrificed on the fifteenth day (Montez et al., 2005). For all other studies, mice were sacrificed after twelve days of either saline or leptin infusion (Cohen et al., 2002). Pair fed animals were implanted with a PBS pump, and fed the same amount of food that their leptin treated counterparts ate on the same day.

Isolation of Mitochondria from Liver

Mice were sacrificed by cervical dislocation and mitochondrial isolation was carried out according to standard procedures (Chappell and Hansford, 1972). Of note, it was important not to use CO_2 to sacrifice the mice, as doing so may uncouple the mitochondria and greatly affect any assays, including differential centrifugation. Immediately after sacrifice, livers were excised and rinsed with ice-cold STE buffer

(250mM Sucrose, 5mM Tris, 2mM EGTA, pH to 7.4). In a beaker, the livers were chopped with scissors and washed with STE until all the blood was removed. They were then homogenized in a glass Dounce homogenizer using 7 strokes of a loose pestle, followed by 3 strokes of a tight pestle, in approximately 40ml of STE. The homogenate was poured into a falcon tube and centrifuged at 1,047 x g for 3 min at 4° C. The supernatant was transferred to a new falcon tube and centrifuged at 11,630 x g for 10 min at 4° C. The supernatant was discarded and the pellets were gently re-suspended with a re-suspending stick. Care was taken not to disturb any blood spot. STE was added to the re-suspended pellets, and the suspension was transferred to a clean falcon tube and spun for 10 mins at 11,630 x g. The previous three steps of discarding the supernatant, re-suspension and centrifugation, were carried out once more. Finally, the mitochondrial extract was re-suspend in approx 200ul of STE.

Purification of Liver Mitochondria

In the case of liver mitochondria being used for iTRAQ protein analysis, further purification was done using a 5% to 35% continuous gradient of nycodenz (Sigma D2158) made in Solution B (0.22M Mannitol, 70mM Sucrose, 1mM EDTA, 10mM MES, pH 7.5). Mitochondria isolated by the method described above were re-suspended in Solution B instead of STE in the last step, and loaded onto a continuous nycodenz gradient [made by mixing 5% and 35% nycodenz in Solution B using a gradient maker]. The tubes were then centrifuged at 75,000 x g in a Beckman SW41

Ti swinging-bucket rotor for 2 hours at 4° C). The mitochondrial band was removed and assayed for protein content using a 2-D Quant Kit (Amersham Biosciences, NJ).

Western Blotting to Assess Mitochondrial Purity

A 12% SDS gel was prepared as follows (recipe for 2 gels): (a) Separation gel – 2.5ml 40% Acrylamide (29:1), 2.5 ml 4x Buffer, 4ml H_20/Glycerol (10:8), 37.5ul 10% APS (fresh or frozen), 7.5ul Temed (b) Stacking gel – 0.65ml 40% Acrylamide (29:1), 1.25ml 4x Buffer, 3.05ml H_20, 25ul 10% APS (fresh or frozen), 5ul Temed. The separation gel was poured into the chamber first, adding the stacking gel and comb afterwards, and was allowed to sit for 15 mins to polymerize. Next, the chamber was filled with running buffer. The gel was run at 70V until the samples made it through the stacking gel and were in one straight line, after which the voltage was increased to 140V. The gel was run at this voltage for approximately 1.5 hours (depending on the percentage of gel).

The sandwich for blotting was prepared by putting the following on the white side (positive side) of the sandwich maker: a) pad soaked in running buffer, b) whatman paper, c) membrane, d) gel, e) whatman paper, f) pad soaked in running buffer. The chamber was filled with blotting buffer and run in the cold room for three hours at 38V. An ice holder and a stir bar were placed in the chamber to keep the temperature down. After it had been run, the membrane was blocked for 30-60 mins with 0.75g of fat-free powdered milk in 15ml of TBS-T. It was then washed with TBS-T three times for 5 minutes each cycle.

The 1° antibody was made by adding 0.75g of fat-free powdered milk to 15ml of TBS-T and the appropriate amount of 1° antibody. The membrane was incubated with this mixture overnight at 4°C. The next morning, the membrane was washed with TBS-T three times for at least 8 minutes each cycle. The 2° antibody was then made by adding 0.75g fat-free powdered milk to 15ml of TBS-T and the appropriate amount of antibody. The membrane was incubated with this mixture for 30min at room temperature. After the incubation, the membrane was washed with TBS-T four times for 15 mins each cycle. Finally, the membrane was dried shortly by waving it in the air.

Equal volumes of detection reagent 1 and detection reagent 2 (for a total volume of 750ul for each membrane) were added to the membrane, and it was covered with saran wrap. In wrapping the membrane care was taken to make sure there were no air bubbles. The membrane was incubated in this mixture for one minute, removed, and dried shortly by waving in the air. Next, the membrane was covered in saran wrap and taped to the top left corner of an exposure cassette. The film put in the cassette was exposed to the film for 30sec, 1min, 2min, and 5min on each of the four corners. Finally, the film was put in the developer.

Isolation of Mitochondria from Skeletal Muscle

Mice were sacrificed by cervical dislocation and as much tissue as possible was harvested from the hamstrings, calf, quadriceps, triceps, and pectoral muscles. As it was being collected, the skeletal muscle tissue was placed in CPI medium (0.1M

KCl, 0.05M Tris-HCl, 2mM EGTA, pH 7.4) on ice. At the end of the collection, the medium was poured away, the tissue was weighed (should have approx 11-12g tissue / 5 mice), and more CP1 medium was immediately added to the tissue. A small amount of tissue was taken and cut with sharp scissors to the smallest pieces possible. This was repeated until all the tissue has been minced in this way.

The tissue was then chopped further with scissors in a beaker. The muscle was allowed to sink to the bottom, and the supernatant containing fat, connective tissue and hair at the top was discarded while being careful not to throw away tissue. The beaker was then refilled with CP1, and the previous step of chopping / discarding was repeated two more times (i.e. three total times of chopping and pouring off). On the last step, instead of adding CP1, 10 milliliters of CP2 (0.1M KCl, 0.05M Tris-HCl, 2mM EGTA, 0.5% BSA(fatted), 5mM MgCl2, 1mM ATP, Protease Type VIII (Sigma P 5380) @ 245.7units/100ml, pH 7.4 @ 4˚C) was added to beaker the for every gram of tissue. It is important not to have the CP2 more dilute as this causes over digestion of the tissue resulting in a poor yield of mitochondria. Keep in mind that CP2 can be made the day before, but the ATP and protease should be added on the day of the experiment and the pH rechecked. Also, the solutions should be dissolved completely before adding MgCl2.

The 250mL beaker with tissue suspended in CP2 was left stirring gently on ice for 3 min (the timing is very important). The muscle was then allowed to sink to the bottom, as much CP2 as possible was poured off, and the beaker was filled with

CP1. The tissue was then transferred into approx 20 round-bottom tubes per preparation on ice. The Polytron was used on each of the tubes for 20 sec, and the supernatant was poured into a 50mL falcon tube. Next, the contents of the falcon tube were transferred to a Dounce homogenizer, and care was taken not to transfer large chunks. Carefully, the tissue was plunged 5 times with the loose plunger, and 3 times with the tight plunger. After each plunge, the chunks of tissue were cleaned off the pestle with a Kimwipe. For large volumes of tissue, the homogenization was done in two shifts.

After the tissue had been homogenized, it was spun at 4˚C for 10 min at a speed of 490 x g. The supernatant was transferred to a new tub and filtered through a 70ul cell strainer to remove debris. It was then spun at 4˚C for 10min at 10,368 x g. Carefully, the supernatant was discarded and the pellet was re-suspended using a "re-suspension stick". The falcon tube containing the sample was then filled with 30ml of CP1 and care was taken to make sure the entire sample had been re-suspended. The solution was then transferred to a new falcon tube and the last spin step was repeated. The supernatant was discarded, the pellet re-suspened, and 180μL of CP1 was added. At the end, it is important to make sure there are no clumps of protein before determining the protein concentration using the Biuret method (Gornall et al., 1949).

Isolation of Mitochondria from Heart Muscle

Mice were sacrificed by cervical dislocation, the hearts were removed and immediately placed in ice cold STE (recipe in previous section). In a beaker, the

hearts were chopped with scissors and washed with STE until all the blood was removed. As much STE as possible was poured off, being careful not to lose any of the minced heart tissue, and was replace with 10mL of STE + 20mg Protease Type VIII (Sigma P 5380). The suspension was left stirring on ice for 2 minutes. The tissue was allowed to sink to the bottom, the supernatant was carefully poured off, and the beaker was filled to 70ML with STE. Next, the contents of the beaker were transferred to a Dounce homogenizer, plunged 5 times with the loose plunger, and 3 times with the tight plunger.

The resulting suspension was centrifuged for 10 minutes at 4˚C at a speed of 8,000 x g. The supernatant was disposed of and the pellet was re-suspend using a "re-suspending stick". All the protease should have remained in the supernatant, thus avoiding over-exposure of the mitochondria to the protease. The suspension was centrifuged for 10 minutes at 4˚C at a speed of 700 x g, the supernatant was decanted into a falcon tube, and the pellet was disposed of. Next, the falcon tube containing the supernatant was spun for 10 minutes at 4˚C at a speed of 8,000 x g. Finally, the supernatant was disposed of, the pellet re-suspended in ice cold STE, and the protein concentration determined using Biuret.

Isolation of Primary Hepatocytes and Measurement of Basal Metabolic Rate

Murine primary hepatocytes were isolated according to a slightly modified version of the method developed by Seglen (Seglen, 1976). The portal vein was cannulated, a suture tied around the cannula, and liver perfusion medium (Invitrogen,

17701-038) with 0.01M HEPES buffer added and warmed to 37°C was perfused through the liver for 5 minutes. Next, collagenase medium (HBBS w/out phenol red from Cellgro, 16mM $NaHCO_3$, 1% FA free BSA, 0.1M HEPES, 0.05% Collagenase Type IV – Sigma C5138, pH 7.6 at 37°C) warmed to 37°C was perfused for 5 minutes. Liver cells were dispersed and gradient centrifuged in isolation media (Cellgro DPBS, 1mM sodium pyruvate, 2% FA free BSA, 25mM Dextrose, 0.03M HEPES, pH 7.6 at 4°C) according to the aforementioned Seglen method. The isolated hepatocytes were switched to incubation medium (106mM NaCl, 5mM KCl, 25mM $NaHCO_3$, 0.41mM $MgSO_4$, 10mM Na_2HPO_4, 2.5mM $CaCl_2$, 10mM glucose, 10mM lactate, 1mM pyruvate, 2.25% (w/v) FA free BSA, pH 7.4 at 37°C), and respiration was recorded as outlined previously (Harper and Brand, 1993). To determine leak and non-mitochondrial respiration, cells were incubated with oligomycin (1.0 ug/ml), and myxothiazol (1.0um).

Measurement of Mitochondrial Respiration

A Clarke-type oxygen electrode (Dual Digital Model 20, Rank Brothers, UK) fitted with a circulating water bath (Model 3016H, Fisher Scientific) was used to measure mitochondrial respiration rate. Mitochondria were suspended in either KHEP (120uM KCl, 5mM KH_2PO_4, 3mM HEPES, 1mM EGTA, 0.3% defatted BSA, pH 7.4) or MRS (70mM Sucrose, 43mM KCl, 3.6mM $MgCl_2$, 7.2mM KH_2PO_4, 36mM Tris-HCl, 0.3% defatted BSA, pH 7.4) at a concentration of 0.5mg/ml and maintained at 37°C throughout the recordings. The oxygen solubility of the medium was

considered to be 406 nmol/ml (Reynafarje et al., 1985). Rotenone (5μM), Succinate (4mM), ADP (1M), Oligomycin (1μg/ml), and FCCP (0.3μM) were added to the recording chamber in a stepwise fashion to measure State II, State III, State IV, and maximal capacity rates. Data were handled and analyzed using a PowerLab 4/20 data recorder and Chart Pro Software (both from AD Instruments, CO).

Modular Kinetic Analysis

The kinetics of proton leak, substrate oxidation, and ATP turnover were determined as described previously (Brand, 1995; Brand et al., 1993; Hafner et al., 1990; St-Pierre et al., 2003). Mitochondrial membrane potential was measured using a methyltriphenylphosphonium ($TPMP^+$) electrode as described earlier (Brown and Brand, 1985), using a $TPMP^+$ binding correction of 0.54. Mitochondria were incubated at a concentration of 0.5mg/ml in KHEP assay medium (composition in previous section), and maintained at 37^{o}C throughout the assay. For proton leak and substrate oxidation kinetics, rotenone (5μM), oligomycin (1μg/ml), and nigericin (0.11 μM) were present at the beginning of each run. For ATP turnover kinetics, rotenone (5μM), nigericin (0.11 μM), and ADP (0.57mM) were present at the beginning of each run. $TPMP^+$ was then added up to 1.3μM for calibration, and succinate (4mM) was fed to the mitochondria as a substrate. Either malonate (proton leak, and ATP turnover) or FCCP (substrate oxidation) was then gradually added to a concentration of 1.3mM or 0.85μM, respectively, to reduce the membrane potential.

At the end of each recording, 1.1μM FCCP was added to determine the drift of the electrode, if any.

Protein isobaric labeling with iTRAQ reagents

Mitochondria were solubilized with 7 M urea, 2 M thiourea and 100 mM N-octyl-β-D-glucopyranoside and protein concentration determined. Protein (60-100 ug) was precipitated with acetone (90% final, v/v) at -20^{o}C overnight. For the four-plex isobaric labeling, separate mitochondrial preparations containing equal amounts of protein were treated in parallel essentially as described by (Ross et al., 2004). Stock reagents and buffer (TEAB, SDS, TCEP, MMTS and four isobaric tagging reagents) are obtained in kit form (Applied Biosystems). Protein (60-100 μg protein) was suspended in 20 ul 0.5 M TEAB, reduced with 2.5 mM TCEP (40^{o}C for 1 h) and cysteine residues blocked with 10 mM MMTS (room temperature for 15 min). Proteolytic digestion was with trypsin (porcine modified, Promega; 1:20, w/w) for 20 h at 40^{o}C. Isobaric tagging iTRAQ reagent (1 unit[1] in ethanol) was added directly to the protein digest (70% ethanol, v/v, final) and the mixture incubated at room temperature for 1h. The reaction was quenched by addition of 9 volumes 0.1% TFA in water (Optima grade, Fischer Sci.). The reaction mixtures are combined and stored at -20^{o}C. Mitochondrial protein from wt (sham), wt (leptin treated), ob (sham) and ob (leptin treated) animals were isobarically labeled with 114.1, 115.1, 116.1 and 117.1 iTRAQ reagent, respectively, in all experiments.

SCX Peptide Fractionation

The iTRAQ four-plex-labeled peptide mixture (400, 252 and 276 μg digest for experiments A, B and C, respectively) was applied to a pair of sulfoethyl aspartamide SCX spin columns (SEM HIL-SCX, PolyLC, The Nest Group, Inc. Southboro, MA) equilibrated with 10 mM KH_2 phosphate, pH 4.5, 20 % CH_3CN. For peptide adsorption to the column and subsequent washing and elution steps, a centrifugal force of 400 x g was used. Excess reagent was washed from the column with 800 μl equilibration buffer. Peptides were eluted with eight 50 μl volumes of KCl in a stepwise gradient from 50-500 mM KCl in equilibration buffer (i.e. 50, 80, 115, 155, 180, 205, 350 and 500 mM KCl) and fractions were dried in a vacuum centrifuge.

Reverse Phase LC

Peptides in SCX fractions were separated by C_{18} nano LC using an 1100 Series nano HPLC equipped with μWPS autosampler, 2/10 microvalve, MWD uv detector (214 nm) and Micro-FC fraction collector/spotter (Agilent). Each SCX salt step was reconstituted with 43 ml 0.1 % TFA, v/v in water and 40 μl injected. Digest was trapped on a C_{18} cartridge (Zorbax300SB, 5 μm, 5 x 0.3 mm; Agilent), desalted with solvent C (CH_3CN:H_2O:TFA, 5:95:0.1) at 20 μl/min for 9 min. and effluent directed to waste. At 9 min post injection,the trapping cartridge was placed ahead of a C_{18} column (Zorbax300SB, 3.5 μm, 150 x 0.1 mm; Agilent) equilibrated with solvent A (H_2O:TFA, 99.9:0.1). Peptides were eluted with a gradient of solvent B (CH_3CN:H_2O:TFA, 90:10:0.1) from 6.5% B to 50% B over 90 min at a flow rate of

0.4 μl/min. Column effluent was mixed (micro Tee, Agilent) with matrix (2 mg/ml α-CHCA in CH_3OH:isopropanol:CH_3CN:H_2O:acetic acid (12:33.3:52:36:0.7) containing 10 mM ammonium phosphate) delivered with a PHD200 infusion pump (Harvard Apparatus) at 0.9 μl/min. Fractions were spotted at 24 sec. intervals onto stainless steel maldi targets (192 wells/plate, Applied Biosystems).

Mass Spectrometry

Mass spectra were acquired on an Applied Biosystems 4800 Maldi TOF/TOF Analyzer (TOF/TOF). MS spectra from 800-3500 Da were acquired for each fraction from 750 laser shots of a 200 Hz YAG laser operated in the 3rd harmonic (355 nm). The TOF/TOF was operated in positive ion reflectron mode. Seven point Gaussian smoothing was applied to spectra and S/N of 30 filter applied for peak picking. Calibration was done using default mode. Plate calibrants were glu-fibrinopeptide A (m/z 1714.787), ACTH (m/z 2753.419), angiotensin (m/z 1440.790), bradykinin (m/z 1048.578), and ACTH 1-17 (m/z 2107.197). The twelve most intense peaks in each MS spectrum were selected for MS/MS analysis. MSMS spectra were acquired from 1500-4000 laser shots using "quality dependent" mode (6 fragment ions at S/N 60); fragment peak picking used S/N 40. MSMS calibration was done with fragments of glu-fibrionpeptide A (m/z 430.242, 684.346, 1056.475 and 1441.634). In a re-interrogation of the target plates, additional MS/MS spectra were acquired. Fragmentation of the labeled peptides was induced by the use of atmosphere as a collision gas with a pressure of ~ 6 x10-7 torr and a collision energy of 2kV.

Peptide identifications were performed using GPS Explorer (v3.6, Applied Biosystems) which acts as a front end for the Mascot search engine (v2.1 MatrixScience, London UK). Each MS/MS spectrum was searched against IPI mouse. Trypsin specificity with one missed cleavage was selected. S-mercaptomethylcysteine and the N-terminal and lysine iTRAQ labels were selected as fixed modifications. Oxidized methionine was considered as a variable modification. The precursor tolerance and MS/MS fragment tolerances were set to +/- 0.7 and +/- 0.3 Da, respectively. A confidence interval (C.I.%) was calculated by GPS Explorer using Mascot ion score and significance, such that a C.I.% value of 95% is equivalent to a Mascot ion score at the significance value. The individual peptide identifications were grouped into protein identifications and assigned a total ion C.I.% by GPS Explorer.

Liver Triglyceride Levels

Total lipids were extracted from liver as described in previously established protocols (Bligh and Dyer, 1959). Quantification of fatty acids was done through liquid chromatography in accordance to previously published methods (Asilmaz et al., 2004; Miyazaki et al., 2001). For the quantification of fatty acids, pentadecanoic acid (Sigma-Aldrich) was added as an internal standard.

Mitochondrial Volume Density and Area Measurements

The unbiased stereological method for the assessment of mitochondrial density was used based upon our published paper (Coppola et al., 2007). The central

feature of this approach is the use of a systematic random sampling method, which meets the statistical requirements necessary to insure an unbiased estimate of the feature of interest. The estimation was performed using the Cavalieri method (Gundersen et al., 1988; Gundersen and Jensen, 1987) and the optical dissector method. The assessment of mitochondrial number was determined using the optical dissector method on electron micrographs. One-way analysis of variance was performed followed by the Student-Newman-Keuls-test to determine significance of differences between groups. The statistical confidence was set at $P < 0.05$.

Lipid Quantification from Purified Hepatic Mitochondria

Samples were isolated by sequential ultracentrifugation. The mitochondrial fraction from each sample was re-suspended in 200 μL of 50% chloroform and methanol (v/v). Twenty μL of sample was analyzed by normal phase HPLC-MS. Lipids were separated on a SUpelcosil –LC-Si column (1 mm * 30 cm; SUPELCO) on the HP1100 HPLC/ABI QSTAR XL LC/MS system, with ion extraction, separation, and detection in both positive and negative ion modes. The solvents were 5 mM ammonium acetate in chloroform/MeOH/(IPA)/water (80:10:7:3) (A) and 5 mM ammonium acetate in MeOH/IPA/water(90:7:3) (B). Data were acquired using information-dependent, automated acquisition. The acquired ms/ms spectra were analyzed to determine the lipid classes and relative quantities.

General Steps and Lipid Extraction for Measurement of Oxidated Fatty Acids

The Hazen Lab at the Cleveland Clinic carried out this procedure in

collaboration with our lab. The methods described in this and the next section discussing measurement of oxidated fatty acids are incorporated from their previously published methods (Zhang et al., 2002). Free fatty acids were purchased from Cayman Chemical Company (Ann Arbor, MI). Organic solvents were obtained from Fisher Scientific Co. (Pittsburgh, PA). All other reagents were purchased from Sigma unless otherwise indicated. All buffers were passed over a Chelex-100 resin column (Bio-Rad) and supplemented with 0.1 mM diethylenetriamine pentaacetic acid (DTPA) to remove potential contaminant transition metal ions that might catalyze lipid oxidation. Protein content was determined using Bradford-based Bio-Rad protein assay using IgG as protein standard.

Lipid extraction was carried out next. All steps were performed under either argon or nitrogen atmosphere. Immediately prior to extraction, 10 ng each of two deuterated internal standards, 12(S)-hydroxy-5,8,10,14-eicosatetraenoic-5,6,8,9,11,12,14,15-d8 acid (12-HETE-d8) and prostaglandin F2 (PGF2 -d4) (Cayman Chemical Company) were added to each sample. Hydroperoxides in samples were then reduced to their corresponding stable alcohols by adding 1 mM SnCl2 (23). Lipids were then extracted by adding a solvent mixture (1 M acetic acid/2-isopropanol/hexane (2:20:30, v/v/v) to the sample at a ratio of 2.5 ml of solvent mixture/1 ml of sample, vortexing, and then adding 2.5 ml hexane. Following vortex and centrifugation, lipids were recovered in the hexane layer. Peritoneal lavages were re-extracted by addition of an equal volume of hexane, followed by

vortex and centrifugation. The combined hexane layers were dried under N2 flow and then analyzed following saponification to release all esterified fatty acid oxidation products. For saponification, N2-dried lipids were resuspended in 1.5 ml of 2-isopropanol, and then fatty acids were released by base hydrolysis with 1.5 ml of 0.2 M NaOH at 60 °C for 30 min under argon atmosphere. Hydrolyzed samples were first acidified to pH 3.0 with 0.5 M HCl, and then fatty acids were extracted twice with 4 ml of hexane. The combined hexane layers were dried under N2 flow, resuspended in 100 l of methanol, and stored under argon at 80 °C until analysis by reverse phase high performance liquid chromatography (HPLC) with on-line electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS), as described in the next section.

Lipid Analysis for Measurement of Oxidated Fatty Acids

LC/ESI/MS/MS was employed to quantify the multiple distinct oxidation products of arachidonic acid and linoleic acid, including individual HETEs, F2-isoprostanes, and hydroxy-octadecadienoic acids (HODEs). Immediately prior to analysis, one volume of H2O was added to 20 volumes methanol-suspended sample, which was then passed through a 0.22- m filter (Millipore Corporation, Bedford, MA). Sample (50 l) was injected onto a C-18 column (2 250 mm, 5 m ODS) (Phenomenex, Rancho Palos Verdes, CA) at a flow rate of 0.2 ml/min. The separation was performed using a gradient starting from 85% methanol over 15 min, then to 100% methanol over 1 min, followed by 100% methanol for 15 min. HPLC column effluent was split so that only 50% was introduced into a Quattro II triple quadrupole

mass spectrometer (Micromass, Inc.).

Analyses were performed using electrospray ionization in negativeion mode with multiple reaction monitoring of parent and characteristic daughter ions specific for each isomer monitored. The transitions monitored were mass-to-charge ratio (*m*/*z*): *m*/*z* 295 3 171 for 9-HODE; *m*/*z* 2953195 for 13-HODE; *m*/*z* 2793261 for linoleic acid; *m*/*z* 3193115 for 5-HETE;*m*/*z* 3193155 for 8-HETE;*m*/*z* 3193151 for 9-HETE;*m*/*z* 319 3 167 for 11-HETE; *m*/*z* 319 3 179 for 12-HETE; *m*/*z* 319 3 175 for 15-HETE; *m*/*z* 303 3 259 for arachidonic acid; *m*/*z* 327 3 184 for 12-HETE-d8; *m*/*z* 353 3 309 for F2-isoprostanes; and *m*/*z* 357 3 313 for PGF2 -d4. The nitrogen curtain gas-flow rate was 5 liters/min and nebulizer gas-flow rate was held at 0.2 liters/min. Collision-induced dissociation was obtained using argon gas. The internal standard 12-HETE-d8 was used to for quantification of HETEs as well as to calculate extraction efficiencies of HODEs and HETEs (which were 85%). The internal standard PGF2 -d4 was used to for quantification of F2-isoprostanes.

Chapter 3: Results

Quantitation of Leptin's Effects on Thermogenesis and Metabolism

Resting core body temperature in leptin deficient *ob/ob* mice is significantly lower than in their wild-type control littermates, and leptin treatment of these mutant mice restores their core body temperature to normal/wild-type levels (Figure 9A). This indicates that a proportion of leptin's metabolic effects in ob mice are a result of increased thermogenesis and subsequent restoration of core body temperature. In order to assess whether there are metabolic effects in addition to this, we treated a second group of ob mice with leptin under thermoneutral conditions. *Ob/ob* mice housed at 30^{o}C, the temperature at which metabolic energy is not required to maintain core temperature (Gordon, 1993), have core body temperatures that are not significantly different from their littermates controls. Leptin treatment of *ob/ob* mice housed at this temperature does not significantly alter core body temperature (Figure 9A).

In order to test whether leptin has additional metabolic effects under these conditions, we compared the effects of leptin treatment to pair feeding of *ob/ob* mice at both 22^{o}C and 30^{o}C. The group treated with leptin at 22^{o}C followed the established trajectory of weight loss (Halaas et al., 1995), and lost fat mass equivalent to 36.5% of their body weight in two weeks (Figure 9B). In comparison, mice pair fed at thermoneutral temperatures lost 13.8% of their body weight during the same time period, indicating that approximately two fifths of leptin-mediated weight loss is due

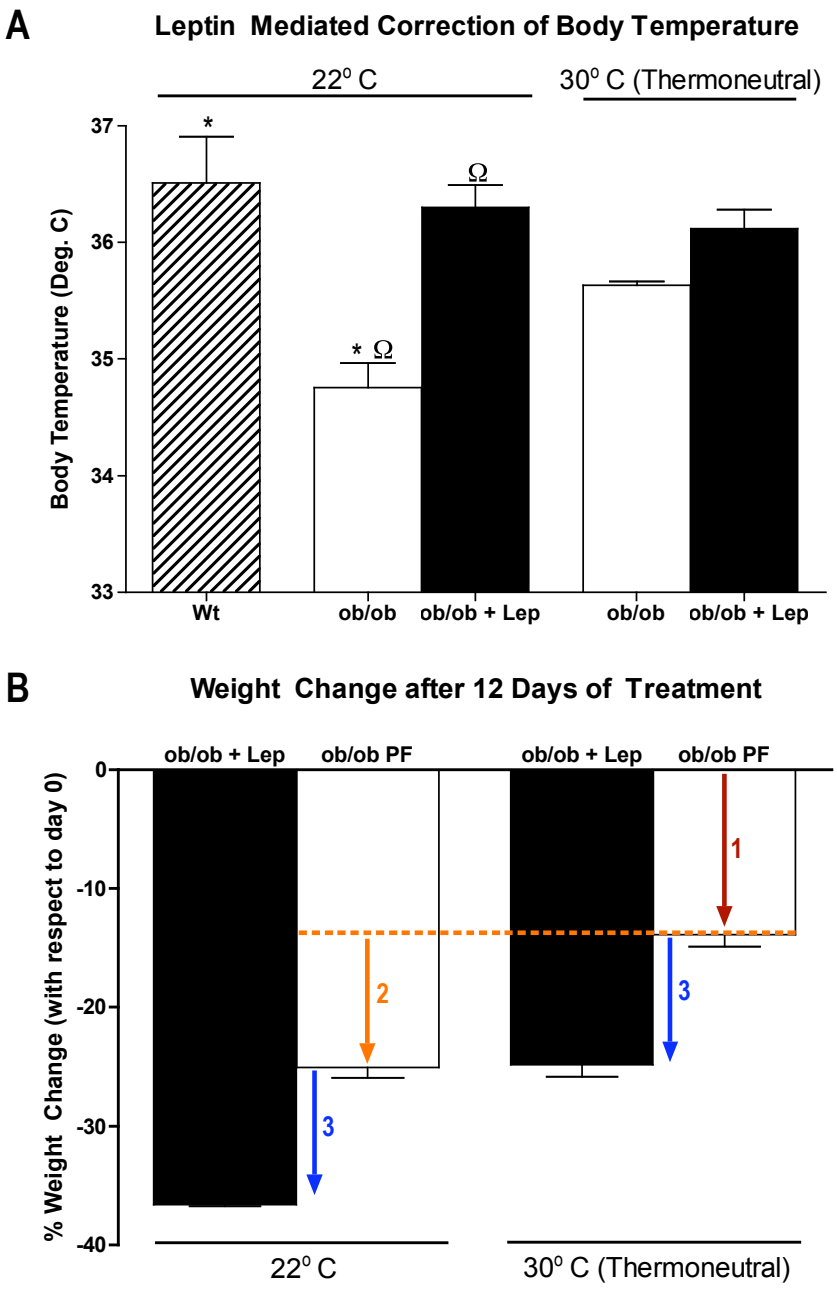


Figure 9. Components of leptin mediated weight loss. (A) ob/ob mice have a lower core body temperature than wt mice, which is corrected by leptin infusion. Housing the mice at thermoneutral temperatures increases the core body temperature of ob/ob mice and diminishes the significance of body temperature correction by leptin. (B) By leptin treating and pair feeding mice at room and thermoneutral temperatures, we were able to distingush the different components of leptin mediated weight loss. Arrow 1 represents weight loss due to leptin's correction of body temperature. Arrow 2 represents weight loss due to leptin's induction of satiety. Arrow 3 represents wight loss due to leptin mediated increases in energy expenditure and basal metabolic rate.

to the induction of satiety (Figure 9B, arrow 1). Leptin treated animals housed at 22^{o}C lost 12% more weight than animals receiving the hormone at 30^{o}C. Similarly, pair fed animals housed at 22^{o}C also lost 12% more weight than animals receiving the same treatment at 30^{o}C. These findings suggest that approximately one third of leptin-mediated weight loss is due to the normalization of body temperature (arrow 2). Another observation we made was that leptin treated animals lost 11% more weight than pair fed animals when housed at 22^{o}C. Leptin treated animals housed at 30^{o}C also lost 11% more weight than their pair fed counterparts. These findings indicate that the remaining one third of leptin-mediated weight loss is due to increases in energy expenditure and basal metabolic rate (arrow 3) ($p<0.0001$ for all comparisons).

Having shown that leptin has metabolic effects beyond normalizing core temperature, we next set out to analyze which tissues contribute to the increased energy expenditure discussed above. We started our investigation with the liver because of several reasons. First of all, the organ plays an integral role in lipid metabolism (Bell, 1979). Secondly, the liver is responsible for approximately 20% of the standard metabolic rate (SMR), and thus has significant energy demands (Porter and Brand, 1993). Finally, the liver is known to be an integral player in some of the catabolic effects of leptin (Cohen et al., 2002). Therefore, the liver made a good candidate organ for the location of the changes in metabolism we were interested in investigating further.

Livers from ob mice are markedly steatotic and 12 days of leptin treatment at ambient temperature corrects this abnormality (Figures 10A and 10B). In order to determine the mechanisms by which leptin carries out this correction, we measured the liver triglyceride (TG) levels of leptin treated and pair fed ob mice housed at both 22^{o}C and 30^{o}C. Animals receiving leptin treatment at 22^{o}C had liver triglyceride levels of 439μM/g, as opposed to 670μM/g for those treated with the hormone at 30^{o}C (Figure 10C). Likewise, animals pair fed at 22^{o}C had liver triglyceride levels of 664μM/g, as opposed to 906μM/g for those receiving the same treatment at 30^{o}C. These data suggest that the steatosis was in part corrected by leptin-mediated normalization of body temperature ($p<0.01$) (Figure 10C, arrow1). Furthermore, leptin treatment at 22^{o}C reduced liver triglyceride levels by approximately 225μM/g over pair feeding at the same temperature (439μM/g vs. 664μM/g) (Figure 10C). Similarly, leptin treatment at 30^{o}C reduced liver triglyceride levels by approximately 236μM/g over pair feeding (670μM/g vs. 906μM/g). These findings indicate that the steatosis is also corrected in part by leptin-mediated increases in energy expenditure and basal metabolic rate ($p<0.01$).

Leptin Levels Influence Hepatocyte Metabolism

Since our data showed a role for leptin-mediated increases in energy expenditure in the liver, we wanted to see if we could measure such changes. Therefore, we analyzed the effects of leptin pre-treatment on freshly isolated primary hepatocyte metabolism. Each day recordings were made from one *ob/ob* + lep animal

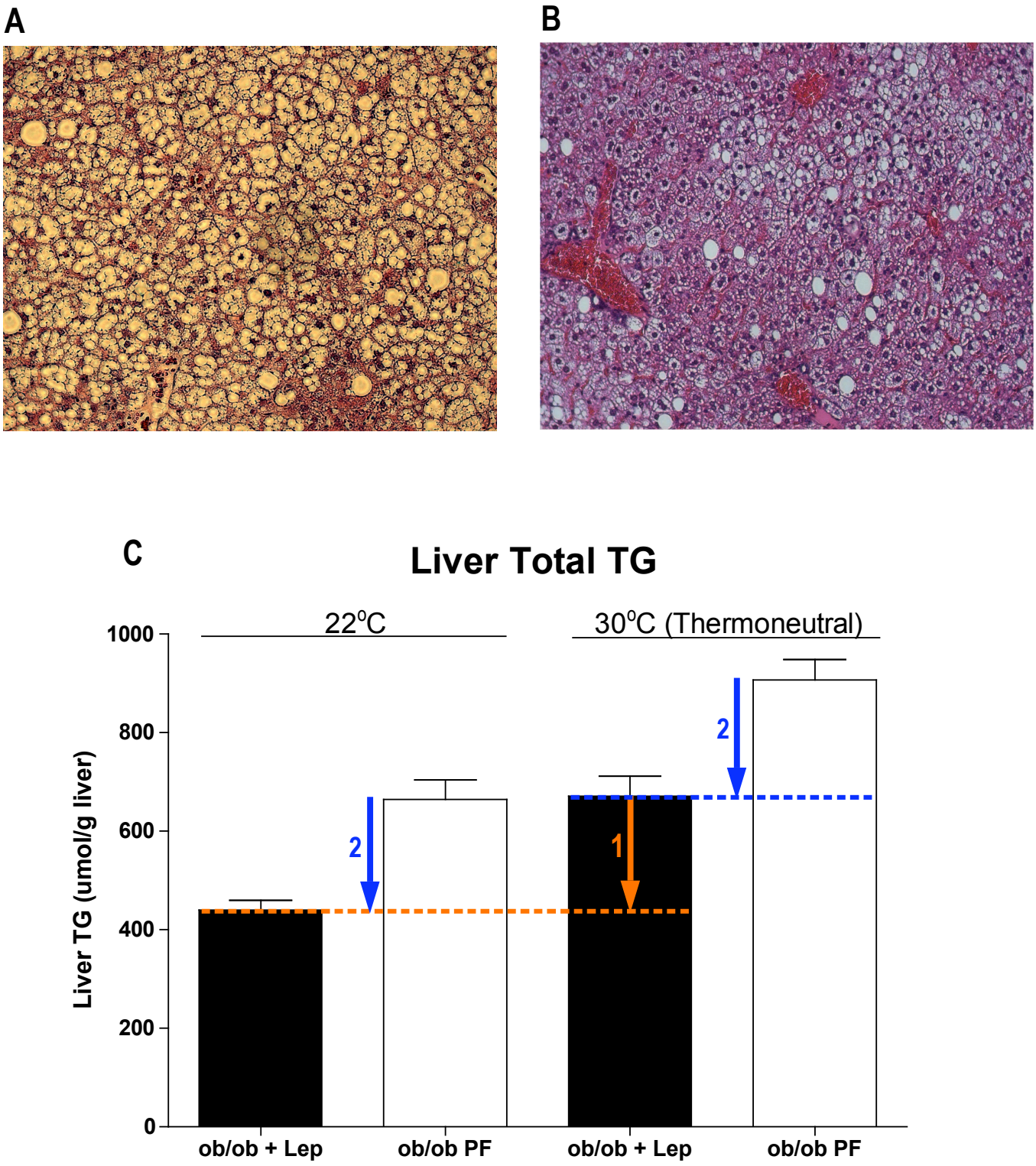


Figure 10. Leptin-Mediated Correction of Hepatic Steatosis. (A) Ob/ob mice have livers exhibiting severe steatosis, which is amended by leptin restoration for 12 days (B). (C) Leptin-mediated correction of hepatic steatosis has one component which is correlated with leptin's correction of body temperature (Arrow 1), and another with leptin-mediated increases in energy expenditure (Arrow 2).

and one *ob/ob* + pbs animal. To correct for daily variances in recordings due to differences in the freshly made solutions, membrane permeability, and variable precipitation on the electrodes, we normalized the data by dividing the values from both groups by the total respiration recorded for that day. This gave us a "normalized ratio" that we could use to compare the rates between the leptin treated and pbs treated groups on different days.

Twelve-day leptin treatment of *ob/ob* mice reduces the primary hepatocyte basal metabolic rate normalized ratio from 0.63 to 0.37 (Figure 11A) ($p<0.0001$). The addition of oligomycin to the solution inhibits Complex V of the mitochondrial electron transport chain, and stops coupled respiration – i.e. respiration that results in the formation of ATP. In this scenario, since coupled respiration has been halted, any respiration that is recorded is due to uncoupled respiration, more commonly referred to as mitochondrial leak. Upon addition of oligomycin in our recordings, the leak component of primary hepatocyte basal metabolic rate is reduced from 64.8% to 41.7% with leptin treatment (Figure 11B)($p<0.05$). These findings indicate that there is a leptin-mediated reduction in the amount of uncoupled respiration. At the end of each recording, we add myxothiazol to determine the non-mitochondrial respiration in the hepatocytes so it can be corrected for in the respiration rates (See methods). However, when we look at the non-mitochondrial respiration itself, we see differences between the two treatment groups. Twelve-day leptin treatment of *ob/ob* mice reduced the non-mitochondrial oxygen consumption normalized ratio in primary

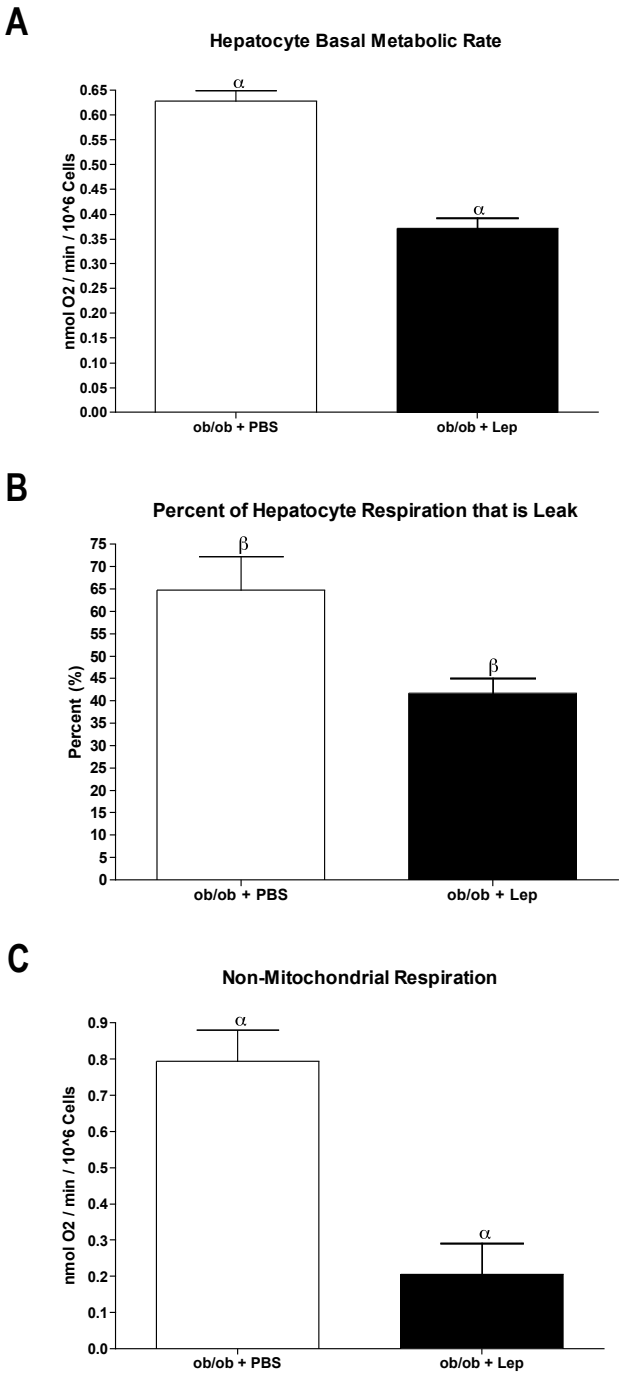


Figure 11. Primary Hepatocytes from ob/ob mice plus/minus leptin. (A) Twelve day leptin treatment of ob/ob mice reduces primary hepatocyte basal metabolic rate. (B) The leak component of basal metabolic rate, i.e. uncoupled respiration, is also decreased with leptin treatment. (C) Finally, leptin treatment decreases non-mitochondrial respiration as well. $\beta = p < 0.05$

hepatocytes from 0.79 to 0.21 ($p<0.01$). Therefore, leptin is having affects on both mitochondrial and non-mitochondrial respiration, which is further explored in the discussion section that follows.

Leptin Inhibits Hepatic Mitochondrial State Two, Four, and FCCP Rates

To test what effects the observed leptin-mediated changes in primary hepatocyte metabolism may be having on mitochondria in these cells, we measured the different components of mitochondrial respiration. Since we had demonstrated that mitochondrial leak was affected in the hepatocytes treated with leptin, we first wanted to look at the respiratory control ratio (RCR) of hepatic mitochondria. This measure is the ratio of coupled respiration over uncoupled respiration (i.e. leak). RCR is lower in *ob/ob* mice (2.43) than in wt mice (4.64) ($p<0.05$) (Figure 12). This difference disappears with leptin treatment of the *ob/ob* animals, as the RCR of leptin treated *ob/ob* mice (5.58) is significantly higher than that of saline treated *ob/ob* mice (2.43) ($p<0.01$).

To determine the cause underlying these changes in RCR, we measured state two, three, four, and respiratory capacity rates. State two and four rates give insight into the leak component of respiration, while state three and respiratory capacity rates provide insight into the coupled component of respiration. Our recordings showed that state two rates were higher in ob mice (55.3 $nmolO_2$/min/mg mito) than in wild-type mice (28.9 $nmolO_2$/min/mg mito)(Figure 13A)($p<0.05$). Leptin treatment of the *ob/ob* mice reduced the state two rates down to 39.8 $nmolO_2$/min/mg mito, and thus

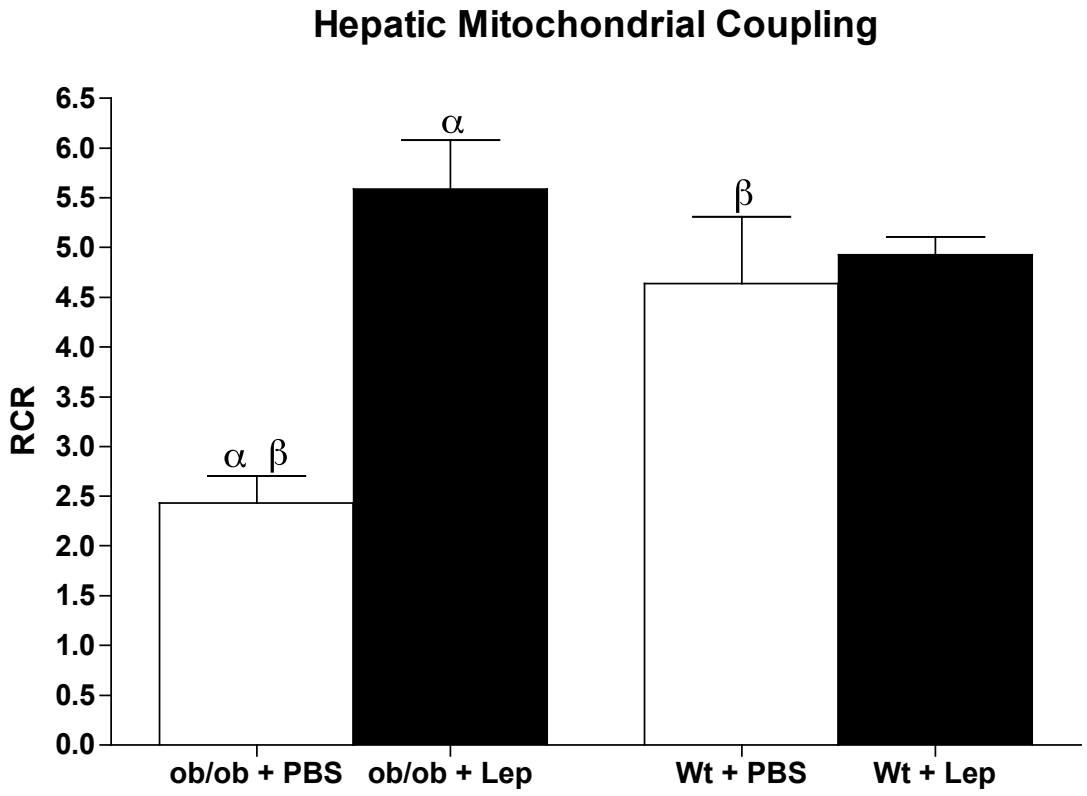


Figure 12. Liver mitochondria RCR analysis from Ob/Ob and Wt mice plus/minus leptin. Respiratory control ratio (RCR) is lower in MRS + BSA media in ob/ob and the effect is reversed by leptin infusion. n=5 for all points, and each was done in duplicate.
$\alpha = p < 0.01$; $\beta = p < 0.05$

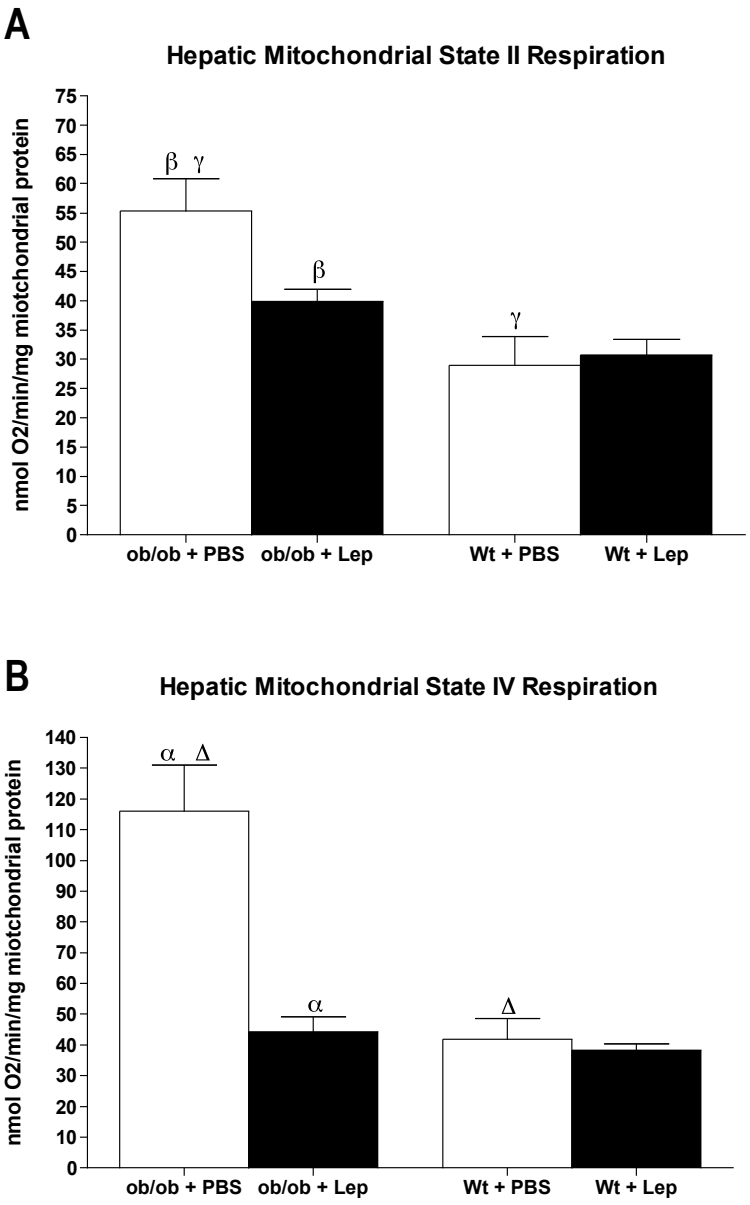


Figure 13. Liver mitochondria leak analysis from Ob/Ob and Wt mice plus/minus leptin. (A) State 2 and (B) State 4 rates are higher in ob/ob mice, which initially suggested that a higher proton leak in ob/ob may be the cause behind the observed changes in the RCR. n=5 for all points, and each was done in duplicate. $\alpha,\Delta = p < 0.01$; $\beta,\gamma = p < 0.05$

diminished the difference between *ob/ob* and wild-type mice. State four rates are also higher in ob mice (116.0 nmolO_2/min/mg mito) than in wt mice (41.9 nmolO_2/min/mg mito). Similar to its effects on state two rates, leptin treatment of *ob/ob* mice reduces state four rates to 44.36 nmolO_2/min/mg mito, thus bringing them to wild-type levels. These initial data suggest that a higher proton leak in *ob/ob* mice may be the cause behind the observed differences in the RCR. Since an increased leak in *ob/ob* animals that is reduced to wild-type levels with leptin treatment is the opposite of what we would have expected, and since both state two or four rates come with caveats regarding their interpretation, we conducted modular kinetic analysis to study these rates in closer detail in the next section.

Having taken a closer look at the denominator of RCR, i.e. uncoupled respiration, we wanted to take a look at the numerator, coupled respiration. As mentioned earlier, state three and FCCP rates provide insight into this component of respiration, and were thus studied next. State three rates are not significantly different between *ob/ob* and wt animals, and do not change with leptin treatment (Figure 14A). Slight differences seem to be present in the means of the rates between the groups, and perhaps with more repeats these would become significant. On the other hand, FCCP rates, which represent mitochondrial respiratory capacity, are higher in *ob/ob* mice (232.8 nmolO_2/min/mg mito) than in wild-type mice (166.0 nmolO_2/min/mg mito)(Figure 14B) ($p<0.01$). Leptin treatment of the *ob/ob* mice reduces FCCP rates to 199.1 nmolO_2/min/mg mito, and thus diminishes the difference between *ob/ob* and

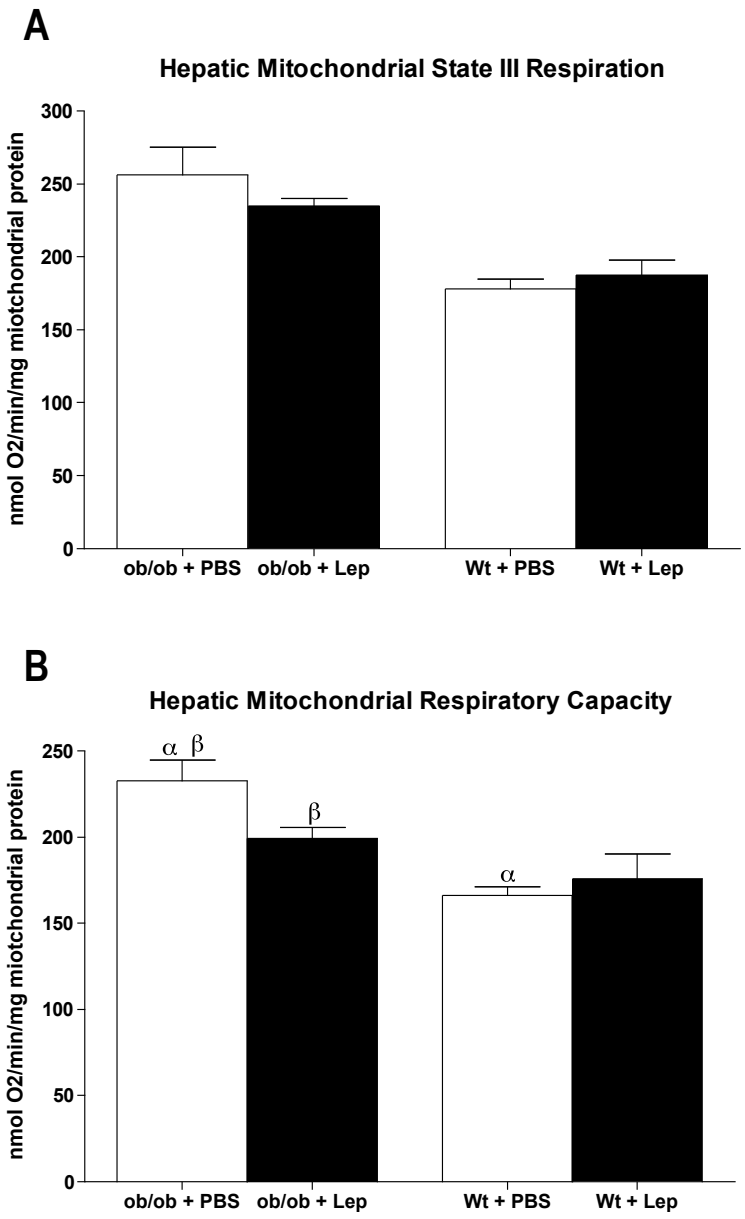


Figure 14. Liver mitochondria substrate oxidation analysis from Ob/Ob and Wt mice plus/minus leptin. (A) State 3 and (B) FCCP rates decrease with leptin treatment, suggesting that the hormone may have effects on the substrate oxidation system. n=5 for all points, and each was done in duplicate. $\alpha,\Delta = p < 0.01$; $\beta,\gamma = p < 0.05$

wild-type animals. These data suggest that *ob/ob* mice have a higher substrate oxidation capacity, which is reduced to wild-type levels with leptin treatment. This possibility is explored further in the next section, where the results of modular kinetic analysis into substrate oxidation capacity are presented.

Since no *in vitro* study can exactly replicate physiological conditions *in vivo*, the artificial environment created by different recording solutions can greatly affect observed mitochondrial respiration rates. Bovine serum albumin (BSA) was present in the recording solution we were using for the recordings described above. The logic behind this was that BSA removes free fatty acids that are in the solution, which can interact with mitochondria to change the inner membrane potential. Though the removal of these free fatty acids is most likely beneficial to our recordings, we didn't know exactly what affect it may be having on the respiration rates. For this reason we did recordings with a solution that was exactly the same as the one used above, except it was missing BSA. In these recordings we found that the respiratory control ratio (RCR) was still significantly lower in *ob/ob* animals as compared to their wild-type littermates (Figure 15A)($p<0.05$). Furthermore, leptin treatment also normalized *ob/ob* rates back to wild type levels in this solution without BSA. Similarly, state two and four rates were significantly lower in wild-type versus *ob/ob* mice ($p<0.05$ and $p<0.01$, respectively), and were both brought towards wild-type levels in leptin treated *ob/ob* mice (Figure 15B and C). Since these results are similar to those

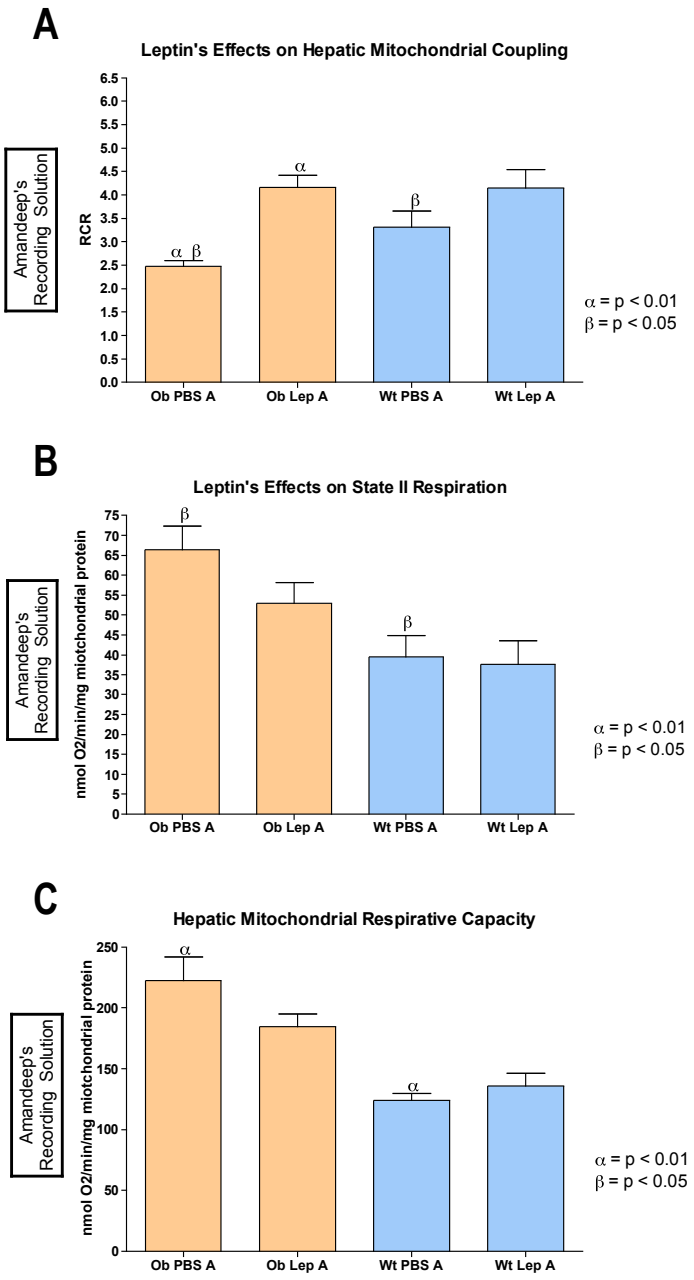


Figure 15. Liver mitochondria respiratory analysis from Ob/Ob and Wt mice plus/minus leptin without BSA in solution. (A) RCR, (B) State II respiration, and (C) FCCP rates are not affected by the presence (Figure 14) or absence (Figure 15) of BSA in the recording solution, suggesting that free fatty acids are most likely not affecting recording rates. n=5 for all points, and each was done in duplicate. α = $p < 0.01$; β = $p < 0.05$

observed in the solution containing BSA, we can conclude that BSA is most likely not affecting our recordings in any significant way.

In our efforts to be thorough with the hepatic mitochondrial recordings, we had come across two commonly used recording solutions. As discussed above, the composition of the solution used can have a great impact on the rates one records, and thus we wanted to see if there were any differences when recording with this second solution. RCR recordings done in the new solution, called KHEP solution (details in methods section), did not show a difference between *ob/ob* mice and wild-type mice (Figure 16A). Furthermore, we did not observe a change in RCR with leptin treatment of *ob/ob* mice when recording from mitochondria in this solution. Similar to the results seen in the other two solutions, state two respiration was significantly lower in wild-type mice as compared to *ob/ob* mice (Figure 16B)($p<0.01$), a difference that was reduced with leptin treatment of the *ob/obs*. Furthermore, FCCP rates were also significantly lower in wild-type mice as compared to *ob/ob* in the KHEP solution (Figure 16C)($p<0.01$). This difference was slightly reduced with leptin treatment, to the point where it became statistically insignificant. Thus in the KHEP solution, RCR rates look different than they do in the other solutions, while state two and FCCP rates look similar to those seen before.

Leptin Inhibits the Hepatic Substrate Oxidation System

To further explore the leptin-mediated changes in mitochondrial leak and substrate oxidation capacity discussed above, we conducted modular kinetic analysis.

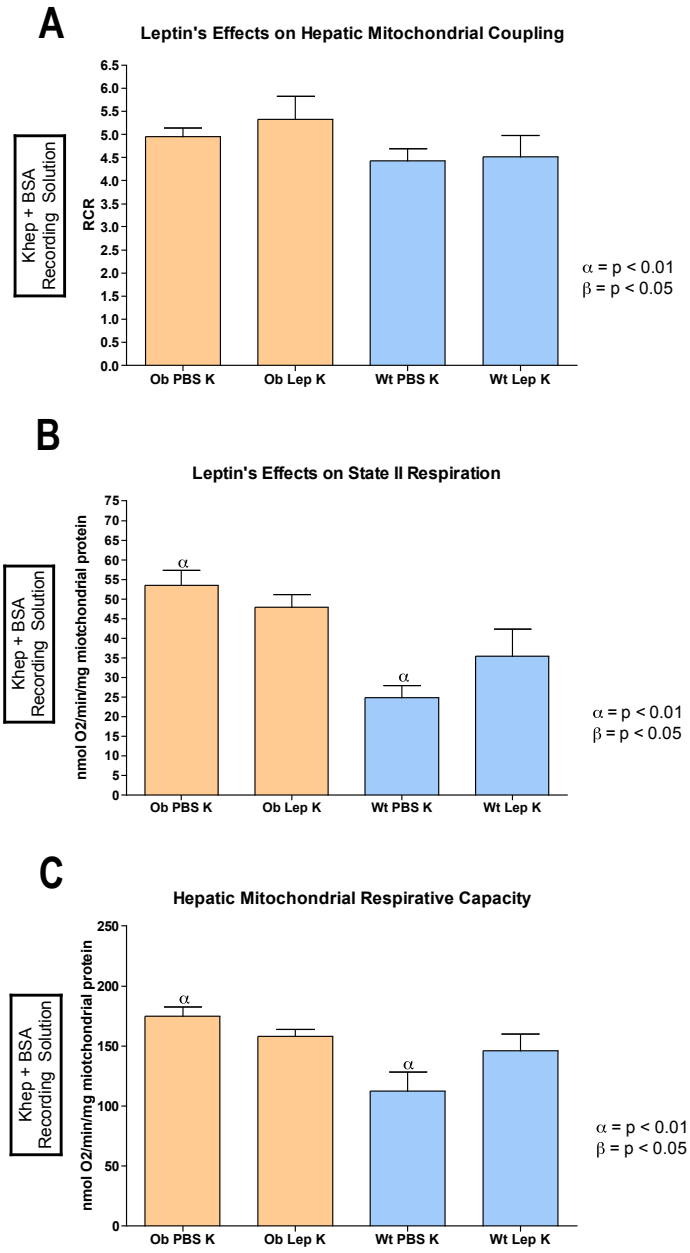


Figure 16. Liver mitochondria respiratory analysis from Ob/Ob and Wt mice plus/minus leptin in KHEP solution. Since there are two mainstream recording solutions used for mitochondrial respiration recordings, we wanted to check if our findings are seen in both. (A) The previously observed leptin-mediated changes in RCR are not seen in KHEP recording solution. (B) State II respiration, and (C) FCCP rates are still reduced with leptin treatment, suggesting that the change in solution had little effect on them.. n=5 for all points, and each was done in duplicate. α = $p < 0.01$; β = $p < 0.05$

In these studies, mitochondrial oxygen consumption is measured with a Clarke electrode, while the mitochondrial inner membrane potential is measured with a TPMP electrode simultaneously (see methods). This allows one to plot changes in respiration as a function of membrane potential, which gives insight into the kinetics underlying mitochondrial respiration rates. Since one can fully appreciate the inner workings of mitochondrial respiration through this method, it is considered to be the "gold standard" of recordings. The difficulties in conducting these recordings attest to the general preference for the use of state two, three, four and FCCP rates instead, which in most cases are sufficiently thorough. In our case, however, we wanted to explore the leak and substrate oxidation findings further, and thus turned to modular kinetic analysis.

In the first set of recordings, we blocked the phosphorylation system with oligomycin, and induced an increase in leak with a titration of FCCP. This causes the proton gradient across the inner mitochondrial membrane to dissipate, and we can see how these changes affect the substrate oxidation system. When we conducted these recordings, we found that the substrate oxidation system is increased in *ob/ob* mice in comparison to wt mice (Figure 17). Twelve-day leptin treatment of these *ob/ob* mice reduces the substrate oxidation capacity of their hepatic mitochondria back towards wild-type levels. These findings can account for the changes in FCCP rates observed in the previous section.

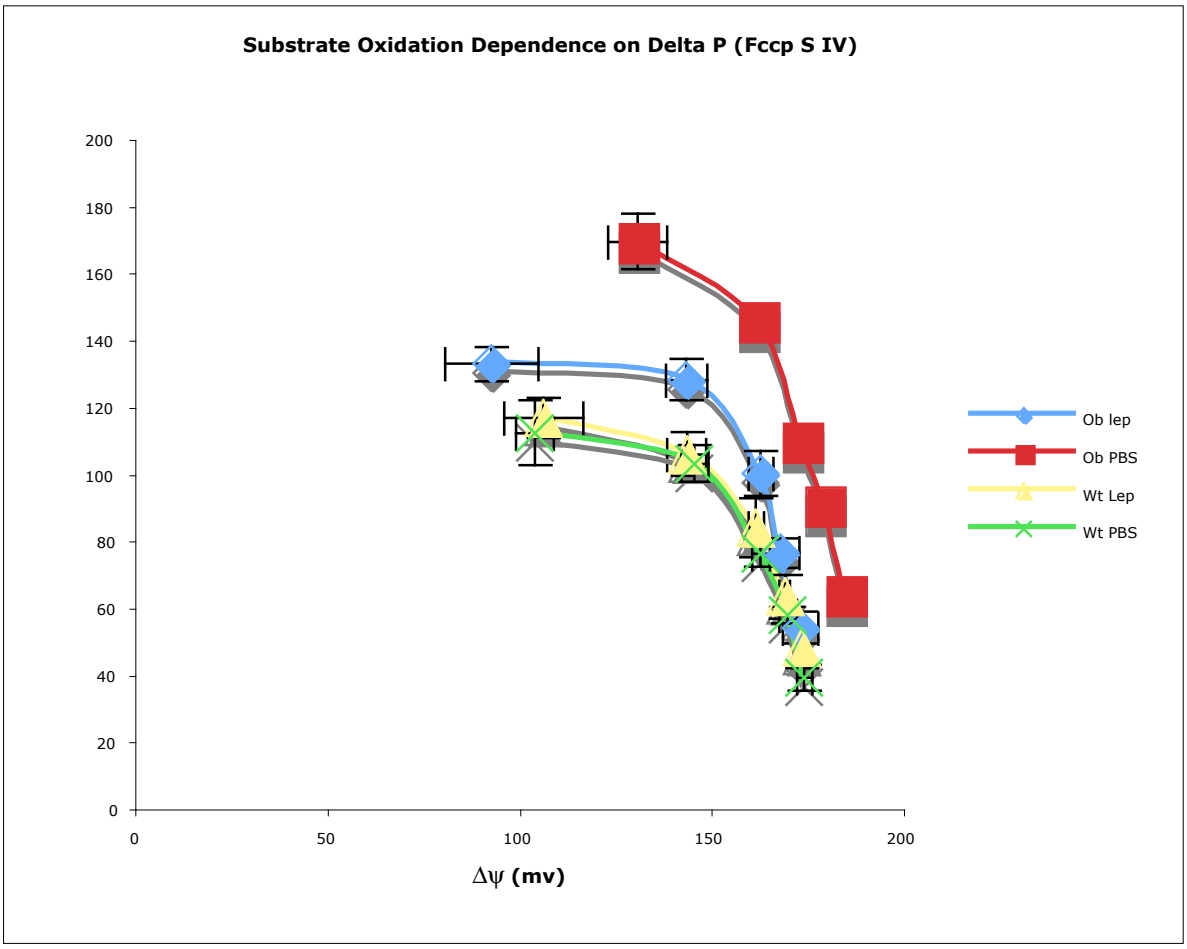
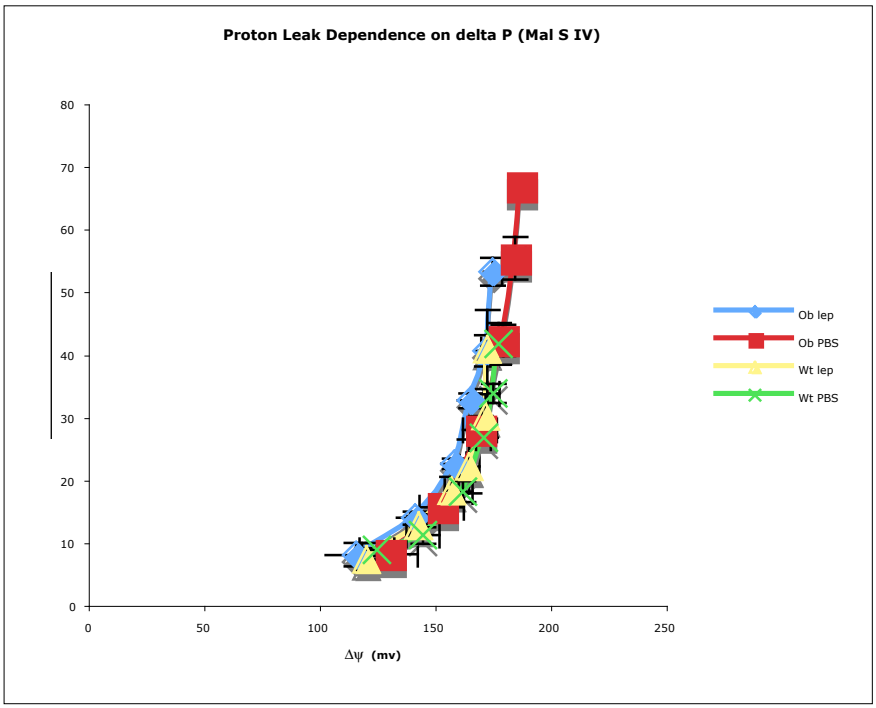


Figure 17. Modular Kinetic Analysis of Leptin's Actions in Hepatic Mitochondria. The substrate oxidation system is increased in ob/ob and partially reversed by leptin. n=5 for all points, each done in duplicate.

In the second set of recordings, we blocked the phosphorylation system with oligomycin like before, but this time we titrated with malonate to block the substrate oxidation system. This again caused the proton gradient across the inner mitochondrial membrane to dissipate, and we could see how these changes affected mitochondrial proton leak. Our recordings show that hepatic mitochondrial leak is not different between *ob/ob* mice and wild-type mice (Figure 18A). Furthermore, leptin treatment does not change mitochondrial leak as the previously recorded state two and four respiration rates had indicated. The reason behind the observed differences is further elaborated upon in the discussion section.

Finally, in the last set of recordings, we just titrate with malonate to block the substrate oxidation system. Once again, this causes the proton gradient across the inner mitochondrial membrane to dissipate. Since there is no blockage of proton leak in this case, our recordings will show the dependence of both leak and the phosphorylation system on the mitochondrial inner membrane proton gradient. By subtracting the leak observations from these recordings, we can study the phosphorylation system in isolation. Our recordings show that the hepatic mitochondrial phosphorylation system is not different in *ob/ob* mice as compared to wild-type mice (Figure 18B). Furthermore, leptin treatment does not affect this system in either *ob/ob* or wild-type mice.

A



B

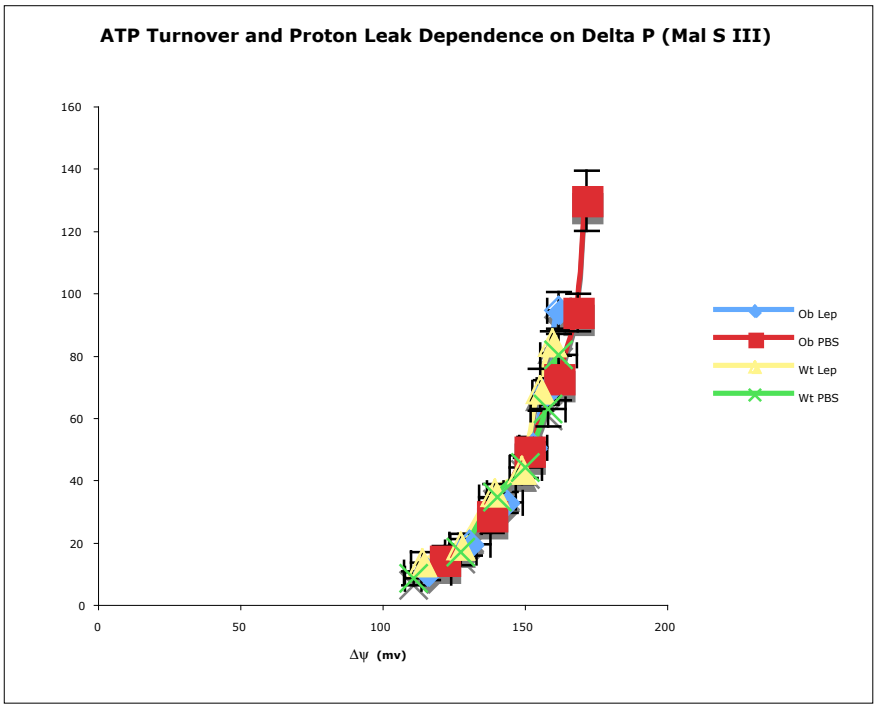


Figure 18. Modular Kinetic Analysis of Leptin's Actions in Hepatic Mitochondria. Neither the leak (A) nor the phosphorylation system (B) change with leptin treatment of ob/ob mice. n=5 for all points, each done in duplicate.

Leptin's Effects on Liver Metabolism Are Independent of its Effects on Hepatic Fat Stores

Leptin's effects on the components of liver metabolism of *ob/ob* mice discussed above could be the result of its ability to correct steatosis and/or a consequence of normalizing circulating leptin levels. To distinguish between these possibilities, we employed a leptin protocol study, in which wild-type mice were given high doses of leptin (2.5mg/hour) for eight days to completely deplete their fat reserves. Upon the cessation of leptin treatment, the mice were divided amongst two groups: free fed (FF) and normo-caloric (NC). The FF animals were allowed to consume as much food as they wanted to for the next seven days - they quickly regained their fat mass and restored normal levels of circulating leptin. Each NC animal was fed the same amount of food it had consumed while on leptin treatment for the seven days after leptin withdrawal - these mice became hypoleptinemic as they could not produce the hormone themselves without adipose tissue. A group of littermates were infused with, and withdrawn from PBS alongside the leptin groups to serve as a further control. Since wild-type mice do not develop hepatic steatosis, this study allowed us to analyze the effects of leptin deficiency independently of any changes resulting from the treatment of hepatic steatosis.

In this study, the RCR was not significantly different in the hepatic mitochondria of hypoleptinemic NC mice as compared to PBS treated controls (Figure 19). Furthermore, the re-establishment of leptin levels in the FF mice did not cause a difference in RCR to arise. These results call for further investigation into

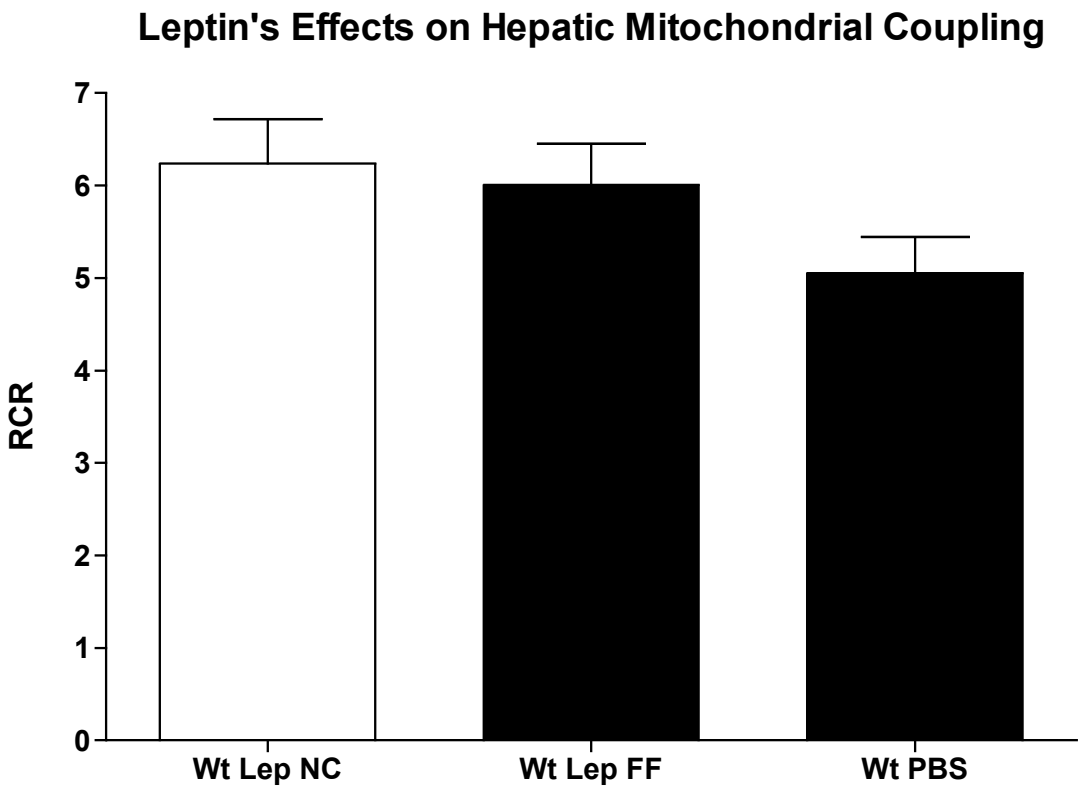


Figure 19. Liver mitochondrial RCR from wt mice with/without hypoleptinemia. To determine whether the observed change in substrate oxidation kinetics (figure 17) is a secondary response to steatosis, attempting to increase ox phos rates in the face of compromised fatty acid oxidation, we looked at miotchondria from mice with hypoleptinemia but not steatosis. Our results show that RCR is not lower in hypoleptinemic "Wt Lep NC" mice, unlike ob/ob mice. This calls for further investigation into the components of RCR, which is shown in the next figure. n=5 for all points, and each was done in duplicate.

coupled and uncoupled respiration rates in order to determine the differences between leptin's actions in the presence or absence of hepatic steatosis.

Looking at uncoupled respiration, we found that state two rates were not significantly different in hypoleptinemic NC mice as compared to PBS treated controls (Figure 20A). Furthermore, these rates were not changed by the re-establishment of circulating leptin levels in the FF mice. Similarly, state four rates were also not significantly different in the NC mice (Figure 20B). Again, the FF mice did not show any differences when compared to both the NC and PBS groups. Thus under conditions where hypoleptinemia does exist but hepatic steatosis does not, there is no evidence of a change in proton leak with leptin treatment. These results are consistent with the data obtained by modular kinetic analysis of *ob/ob* mice in the previous section.

We next turned our attention to the numerator of RCR, i.e. coupled respiration, in these hypoleptinemic mice. State three respiration rates were not different in NC mice versus PBS mice, and leptin normalization in the FF mice did not alter these rates either (Figure 21A). However, similar to leptin treatment of *ob/ob* mice, the oxygen consumption in the presence of FCCP was significantly higher in hypoleptinemic mice (186.1 nmolO_2/min/mg mito) versus that in PBS treated controls (152.0 nmolO_2/min/mg mito)(Figure 21B)($p<0.01$). Furthermore, re-establishment of circulating leptin levels in FF mice reduced the FCCP rates back down to PBS treated levels (154.0 nmolO_2/min/mg mito)($p<0.01$). These data show that in the case of

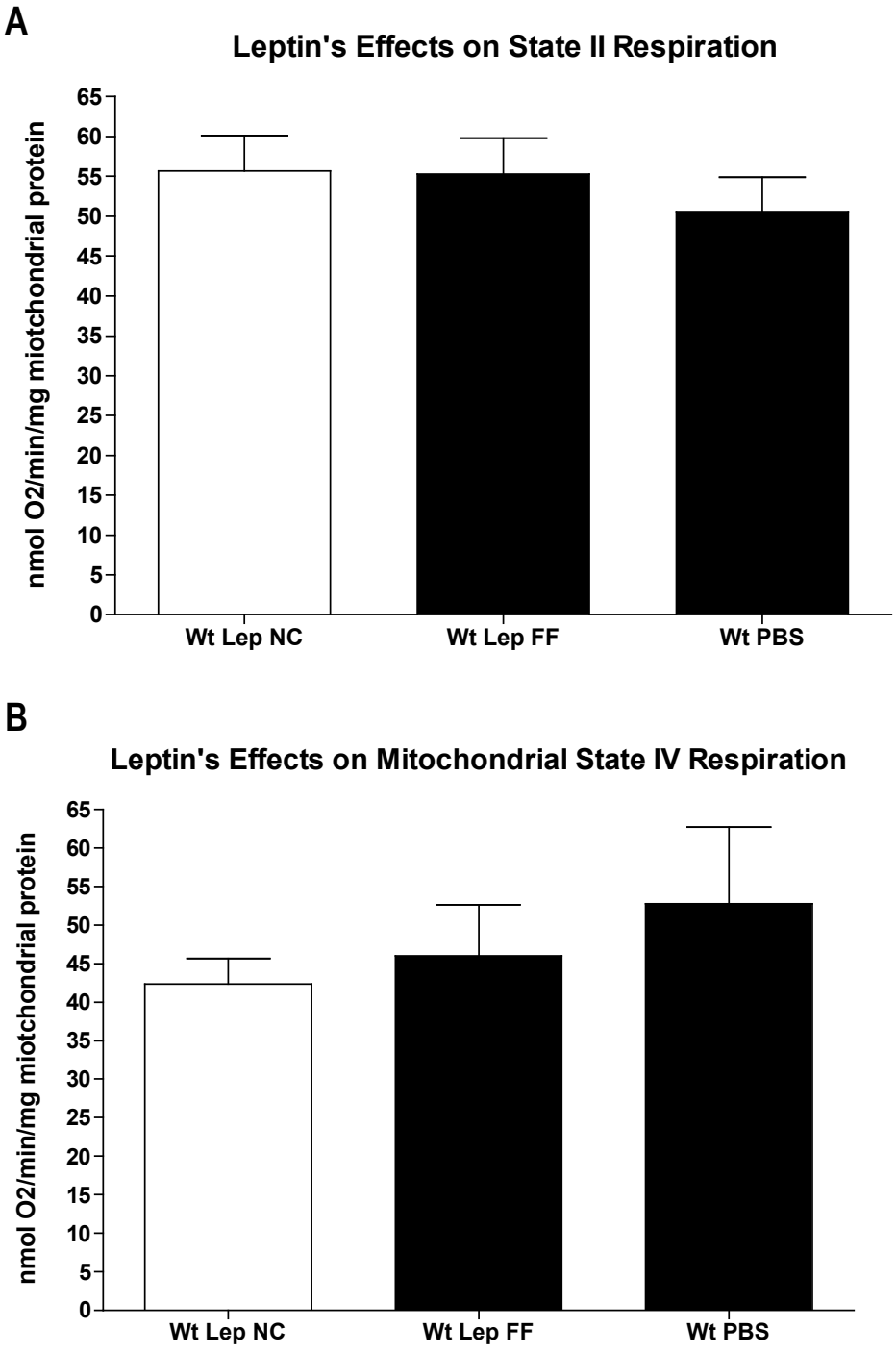


Figure 20. Liver mitochondrial leak from wt mice with/without hypoleptinemia. Neither State 2 (A) nor state 4 (B) rates are different in hypoleptinemic mice, so there is no sign of changes in proton leak. This is similar to observations in ob/obs. n=5 for all points, and each was done in duplicate. $\alpha,\Delta = p < 0.01$

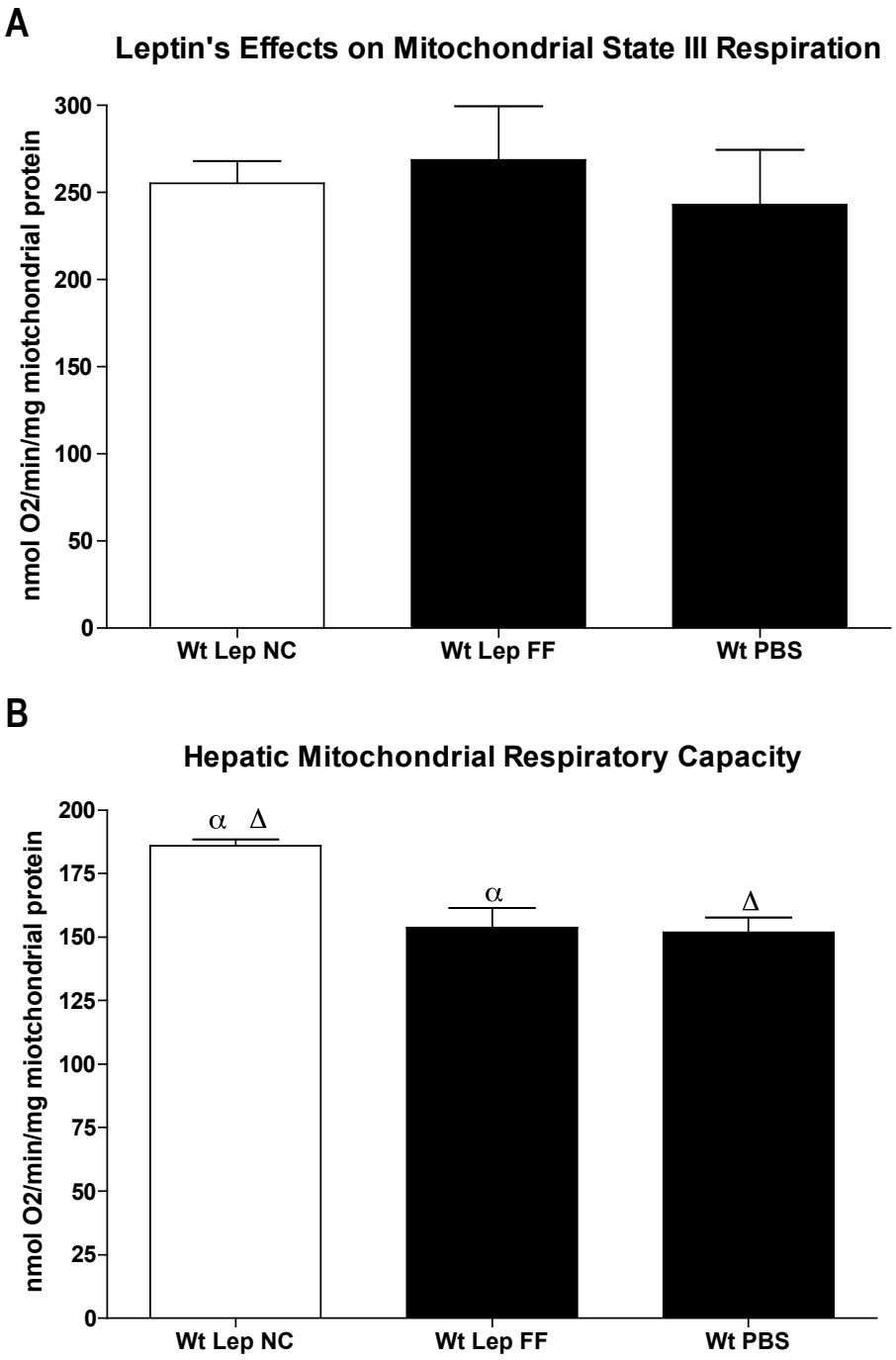


Figure 21. Liver mitochondrial substrate oxidation from wt mice with/without hypoleptinemia. Though State 3 rates do not change (A), FCCP rates are higher in hypoleptinemic mice, and decrease upon restoration of leptin levels (B). This is similar to the leptin-mediated decrease in the substrate oxidation system seen in ob/ob mice. Therefore, we can conclude that leptin's control over the hepatic mitochondrial substrate oxidation system is most likely independent of its effects on hepatic fat stores. n=5 for all points, and each was done in duplicate. $\alpha,\Delta = p < 0.01$

hypoleptinemia without the presence of hepatic steatosis, mitochondrial respiratory capacity is still significantly reduced. Furthermore, re-establishment of leptin levels without any change in hepatic fat stores still significantly decreases the mitochondrial respiratory capacity down towards wild-type levels. Since the effects of leptin in this study on both uncoupled and coupled respiration were the same as what was recorded during modular kinetic analysis of *ob/ob* mice, these data suggest that leptin's effects on hepatic metabolism are not secondary to its ability to reduce hepatic lipid content.

Leptin Levels Influence Mitochondrial Morphology

Mitochondrial morphology is closely linked to the metabolic state of the organelle, and can give great insight into its function. The observation that leptin modulates mitochondrial metabolism led us to next assess mitochondrial morphology and mass before and after leptin treatment using electron microscopy on PBS treated, leptin treated, and pair fed *ob/ob* mice. The mitochondria of 12-day PBS treated *ob/ob* mice have both normal cristae structure and morphology (Figure 22). A total of 54 micrographs from n=5 mice were studied and yielded similar results. Similarly, the mitochondria of 12-day pair fed *ob/ob* mice also had normal cristae structure and morphology (Figure 23). In this case, 53 micrographs from a total of n=5 mice were studied and yielded similar results. Interestingly, the mitochondria of 12-day leptin treated *ob/ob* mice (Figure 24) were morphologically different from both control groups and exhibited a less dense, "ballooned" appearance compared to those of 12-

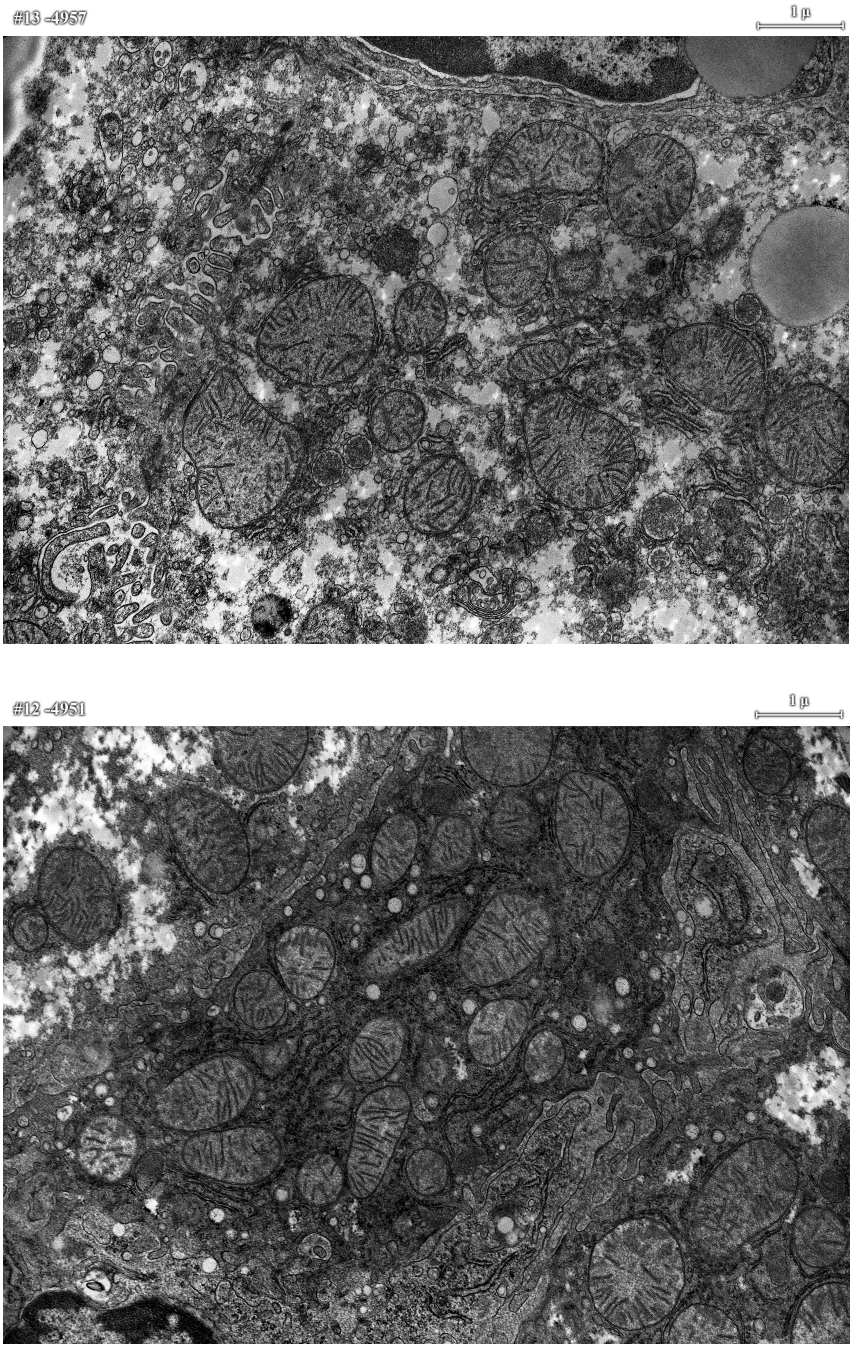


Figure 22. Electron Microscopic Analysis of Hepatic Mitochondrial Structure. Mitochondria in the hepatocytes of PBS treated ob/ob mice have both normal cristae structure and density. A total of 54 micrographs from n=5 mice were studied and yielded similar results.

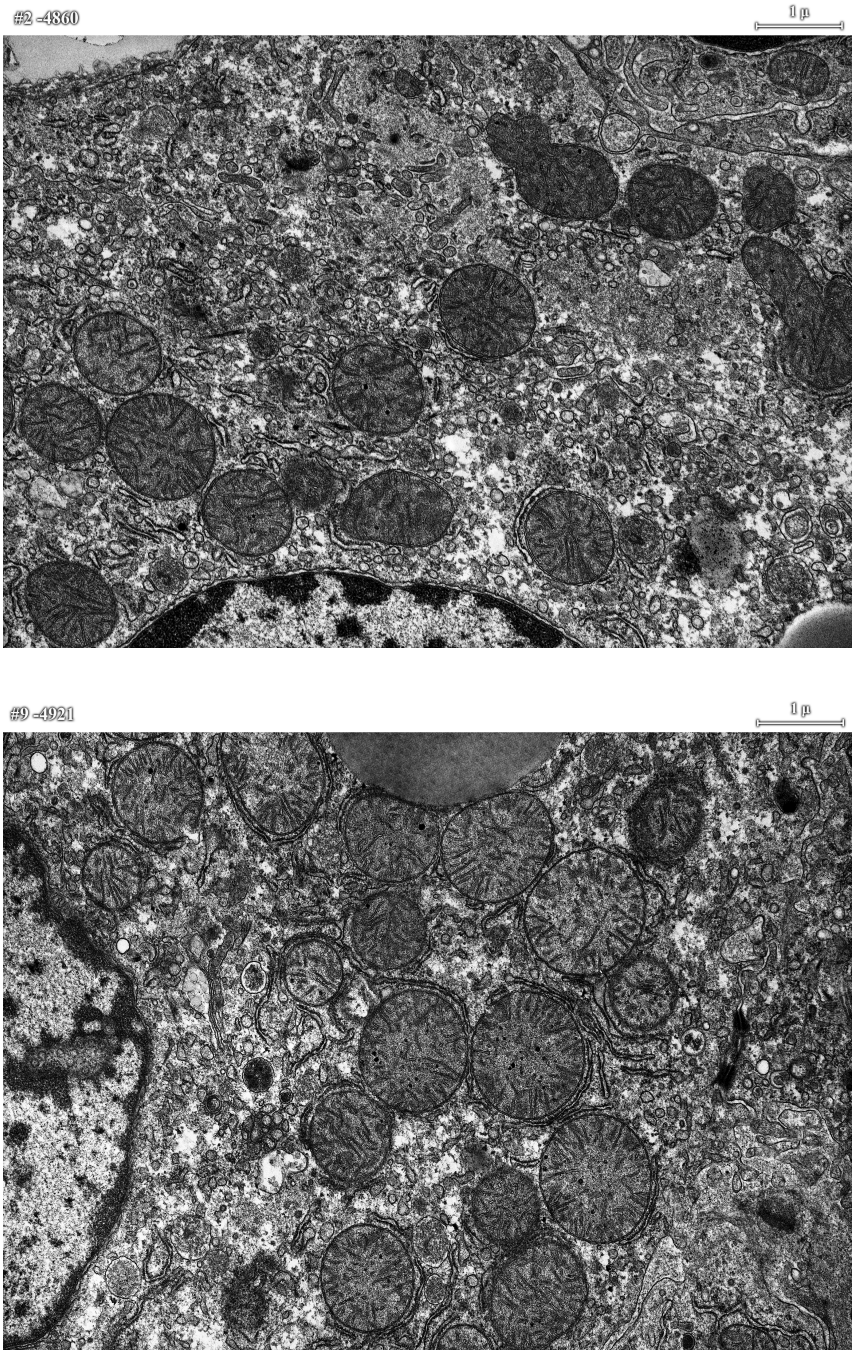


Figure 23. Electron Microscopic Analysis of Hepatic Mitochondrial Structure. Mitochondria in the hepatocytes of pair fed ob/ob mice have both normal cristae structure and density. A total of 53 micrographs from n=5 mice were studied and yielded similar results.

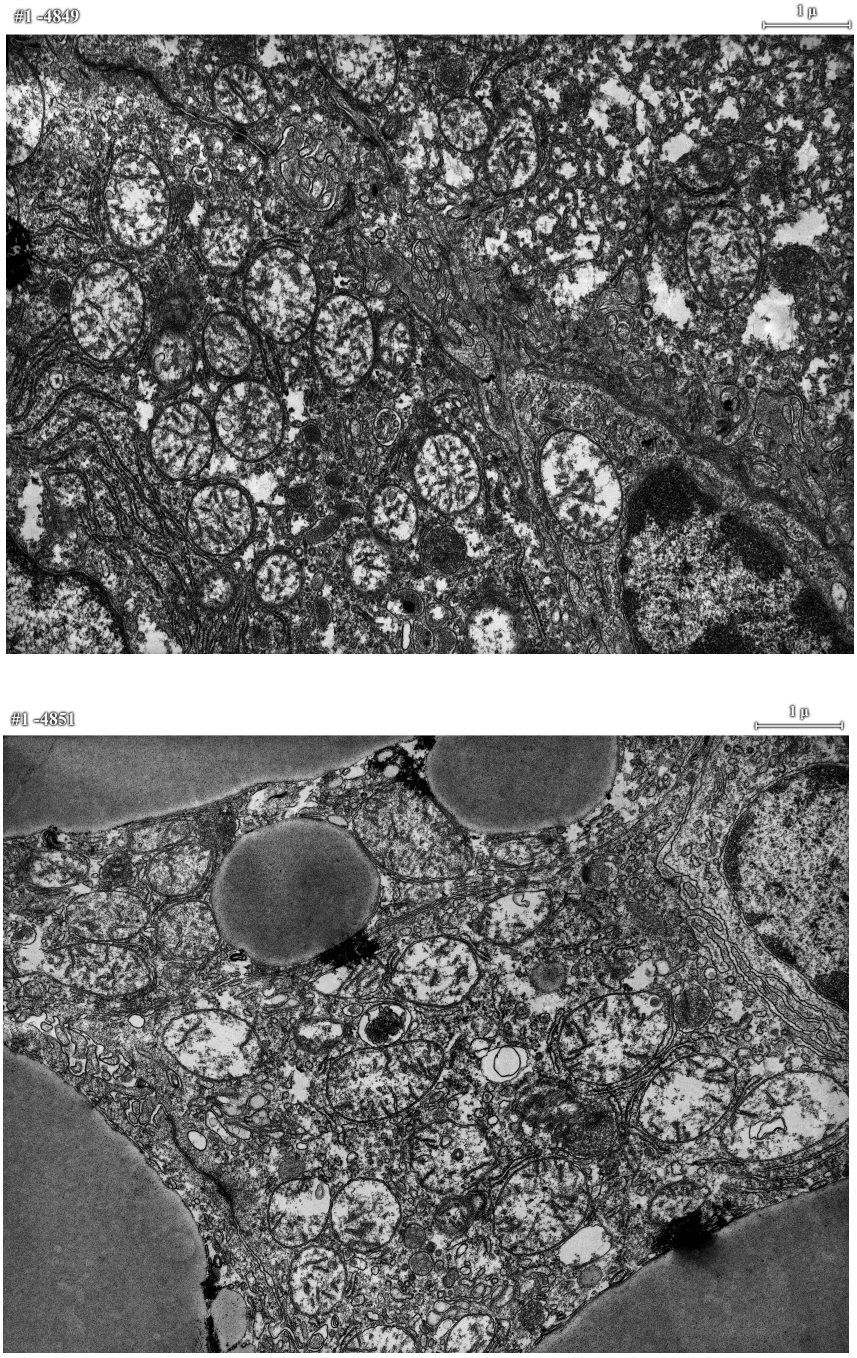


Figure 24. Electron Microscopic Analysis of Hepatic Mitochondrial Structure. Mitochondria in the hepatocytes of leptin treated ob/ob mice look less dense and "ballooned" compared to those from PBS treated (figure 22) and pair fed (figure 23) ob/ob mice. The cristae structure also looks quite different in the leptin treated animals compared to the other groups. A total of 51 micrographs from n=5 mice in this group were studied and yielded similar results.

day pair fed and 12-day PBS treated *ob/ob* mice. The cristae structure was also different in the leptin infused group compared to the others.

Having found structural differences in mitochondrial morphology and cristae structure with leptin treatment, we wanted to see if there were any differences in mitochondrial mass in the hepatocytes. Stereological assessment of these micrographs showed that hepatocytes from leptin treated *ob/ob* animals have a decreased mitochondrial numerical density of 1.57 mito/μm^3 (Figure 25A), in comparison to 2.55 and 2.99 mito/μm^3 for saline treated and pair fed *ob/ob* animals respectively ($p<0.01$ for both comparisons). Furthermore, leptin treatment also decreases mitochondrial volume density in *ob/ob* mice to 0.154 $\mu m^3/\mu m^3$ (Figure 25B), compared to 0.245 and 0.281 $\mu m^3/\mu m$ for saline treated and pair fed *ob/ob* controls respectively ($p<0.05$ for both). Leptin treatment, however, does not alter the average area of each individual mitochondrion with respect to saline treatment (0.90 vs 0.97 μm^2)(Figure 25C). On a side note, it appears that pair feeding increases individual hepatic mitochondrial size in comparison to both leptin treatment and saline infusion ($p<0.05$ for both). These data suggest that leptin treatment leaves each hepatocyte with fewer mitochondria of the same size, the implications of which are explored in more detail in the discussion section.

Proteomic Changes Underlying Leptin's Effects on the Liver

We next set out to investigate whether changes in the levels of specific mitochondrial proteins were associated with the functional and morphologic changes

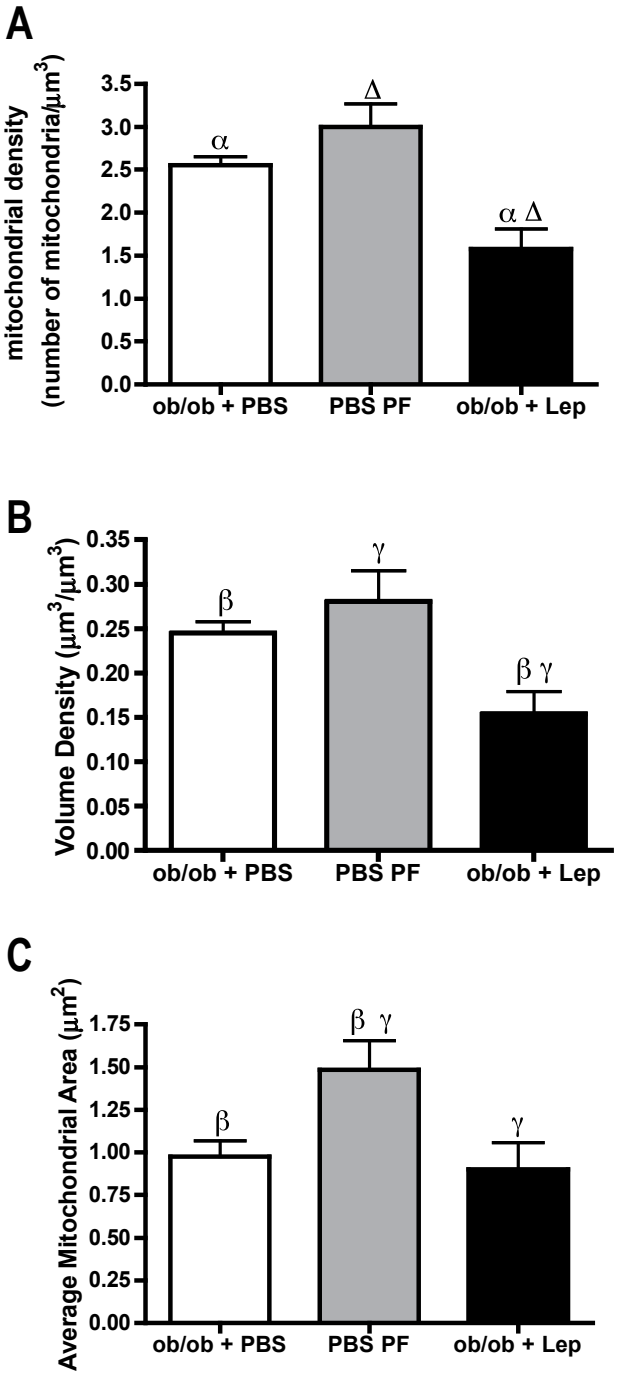


Figure 25. Electron Microscopic Analysis of Hepatic Mitochondrial Morphology. Both mitochondrial numerical density (A) and volume density (B) are decreased with leptin treatment. Theaverage size of each mitochondria, however, is unaffected by treatment with leptin (C). $\alpha,\Delta = p < 0.01$ $\beta,\gamma = p < 0.05$

that were observed. Mitochondria from untreated *ob/ob*, leptin treated *ob/ob*, saline treated wt, and leptin treated wt mice were purified over a nycodenz gradient using a standard centrifugation protocol (see methods). The protein content of equal numbers of mitochondria was quantitated using the iTRAQ system. This method of protein measurement uses four isobaric tags with different reporters, which in tandem mass spectrometry allow one to accurately quantify differences as small as 10% among samples (Keshamouni et al., 2006).

Three proteins that are components of the electron transport chain – cytochrome c oxidase subunit IV, cytochrome c oxidase subunit VIa, and mitochondrial NADH dehydrogenase – were significantly altered by changes in leptin levels (Figure 26). Protein levels of cytochrome c oxidase subunit IV showed no significant difference between *ob/ob* and wild-type mice, but were decreased to approximately 83% of their levels with leptin treatment in *ob/obs* ($p<0.01$). Cytochrome c oxidase subunit VIa protein levels, on the other hand, were found to be increased by 1.23 fold in *ob/ob* mice versus wild type mice ($p<0.05$), and leptin treatment of these ob/obs brought the level of these proteins down to 81% of their normal values ($p<0.05$). Finally, protein levels of mitochondrial NADH dehydrogenase were shown to be 1.53 fold higher in *ob/ob* mice versus wild-type mice ($p<0.05$), and were brought down to 77% of their normal values by leptin treatment in these *ob/obs* ($p<0.05$). The implications of the concerted increase in

ObPBS / WtPBS	ObLep / ObPBS	Protein Name	Cellular Location	Function
No Change	2.43	Acyl-CoA thioesterase 2 (MTE1)	Mito	Hydrolysis of acyl-coas to the free fatty acid and coenzyme a (coash)
1.32	1.52	Aldehyde dehydrogenase family 3, A2	ER	Aldehyde + NAD(+) + H(2)O = Fatty Acid + NADH
1.28	1.32	Medium-chain acyl-CoA dehydrogenase	Mito	Mitochondrial fatty acid beta-oxidation system; first step
1.36	1.28	3-hydroxybutyrate dehydrogenase, type 1	Mito	Interconversion of the two major ketone bodies produced during fatty acid catabolism.
1.34	1.25	Long-chain specific acyl-CoA dehydrogenase	Mito	Mitochondrial fatty acid beta-oxidation system; first step
1.37	0.83	Glutaryl-CoA dehydrogenase	Mito	Oxidative decarboxylation in degradation of L-lysine, L-hydroxylysine, and L-tryptophan
No Change	0.83	Cytochrome c oxidase subunit IV isoform 1	Mito	Terminal enzyme of the mitochondrial respiratory chain
1.29	0.83	3-hydroxyacyl-CoA dehydrogenase type II	Mito	Catalyzes the oxidation of a wide variety of fatty acids, alcohols, and steroids
1.23	0.81	Cytochrome c oxidase subunit VIa polypeptide	Mito	Terminal enzyme of the mitochondrial respiratory chain
1.47	0.81	Acyl-CoA oxidase 1, palmitoyl	Mito	The first enzyme of the fatty acid beta-oxidation pathway
1.35	0.81	Propionyl Coenzyme A carboxylase	Mito	Key enzyme in odd-chain fatty acid, isoleucine, threonine, methionine, and valine catabolism
1.53	0.77	Mitochondrial NADH dehydrogenase 5	Mito	NADH dehydrogenase. Complex 1 of mitochondrial respiratory chain
2.64	0.65	Acetyl-CoA acyltransferase 1B	Mito	Fatty acid metabolism. PPAR signaling pathway
1.47	0.58	Solute carrier family 25 (mitochondrial carrier, citrate transporter), member 1	Mito	Citrate-h+/malate exchange. Provides a carbon source for fatty acid and sterol biosyntheses, and nad+ for the glycolytic pathway.
3.49	0.53	ELOVL family member 5, elongation of long chain fatty acids	ER	Involved in long chain fatty acid elongation. Induction of Elovl-5 leads to massive hepatic accumulation of mono-unsaturated fatty acids

Figure 26. iTRAQ Protein Quantification of Hepatic Mitochondrial Proteins. Three proteins that help comprise the electron transport chain – cytochrome c oxidase subunit IV, cytochrome c oxidase subunit VIa, and mitochondrial NADH dehydrogenase – are significantly altered by changes in leptin levels. The increase in the abundance of these proteins during leptin deficiency and their decrease during leptin infusion may be the mechanism behind the observed differences in hepatic mitochondrial substrate oxidation described earlier. Also intriguing are the differences seen in the levels of the enzyme ELOVL5, which exhibited the highest increase in abundance during leptin deficiency, as well as the greatest decrease in abundance with leptin treatment.

these proteins during leptin deficiency and decrease with leptin treatment is explored in further detail in the discussion section.

Another interesting protein that was found to vary with leptin levels was the enzyme ELOVL5, which had the highest increase in abundance during leptin deficiency, as well as the greatest decrease in abundance with leptin treatment (Figure 26). This enzyme is involved in long chain fatty acid elongation, and its induction has been shown to cause massive hepatic accumulation of mono-unsaturated fatty acids (Wang et al., 2006). ELOVL5 protein levels were increased by 3.49 fold in *ob/ob* mice versus wild-type mice ($p<0.05$), and leptin treatment of these *ob/ob* animals reduced the levels of this enzyme to 53% of its normal abundance ($p<0.05$). The implication of these changes in leptin-mediated correction of hepatic steatosis is explored in the discussion section.

Leptin's Effects on Hepatic Mitochondrial Lipids

Having found significant changes in mitochondrial proteins induced by leptin, we next wanted to see if there were any changes in mitochondrial lipids brought about by the hormone. To do this we purified mitochondria using the same nycodenz gradient centrifugation protocol that was used for protein quantification in the previous section (see methods). Lipids were then eluted and separated by normal phase HPLC from the same number of mitochondria in each sample. The profile of lipid classes obtained by this method is shown in figure 27A. When tandem mass spectrometry (ms-ms) profiles were obtained from these lipids, it became clear that

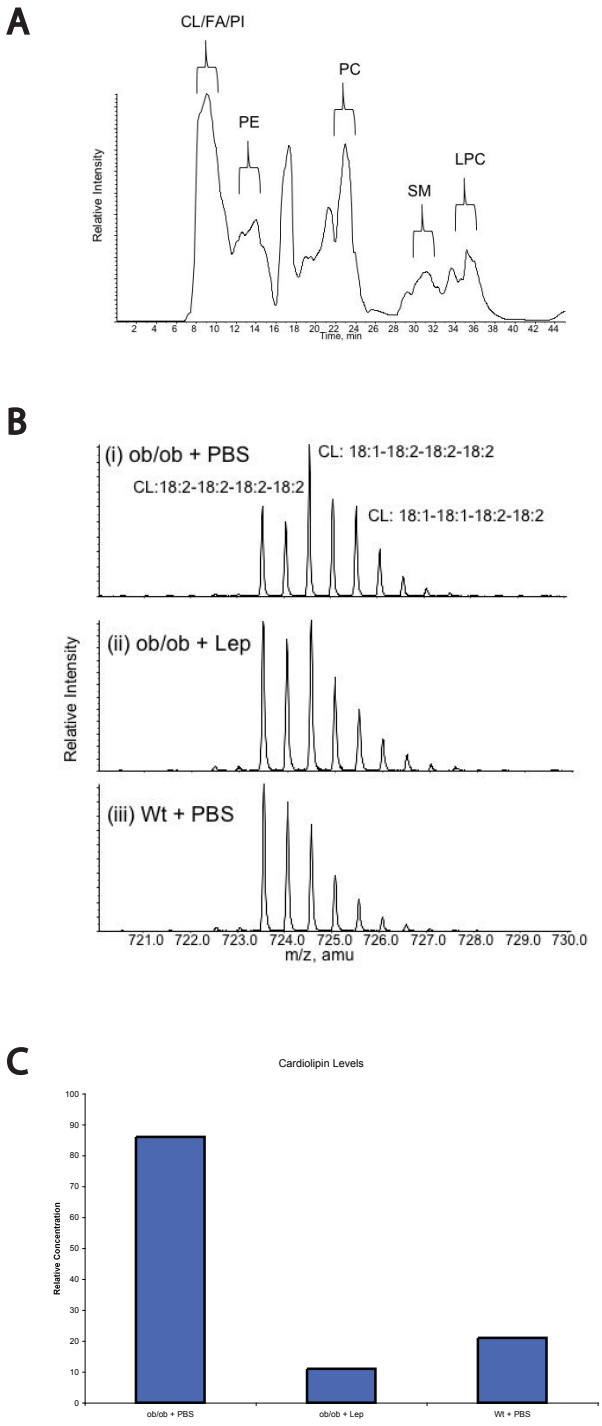


Figure 27. Leptin-Mediated Changes in Hepatic Mitochondrial Cardiolipin Levels. (A) Elution profile of lipid classes separated by normal phase-HPLC (B and C) Cardiolipin levels in hepatic mitochondria are much higher in ob/ob mice than in wild-type mice, a difference that is corrected by leptin treatment. CL: cardiolipin, FA: Fatty Acid, PI: Phosphotidylinositol, PE: Phosphotidylethanolamine, PC: Phosphotidylcholine, SM: Sphigomyelin, LPC: lysophosphotidylcholine

cardiolipin levels were much higher in *ob/ob* + PBS mitochondria than in wt + PBS mitochondria, and this difference was corrected by leptin treatment (Figure 27B). Indeed, quantification of the lipids showed that cardiolipin levels were more than four times higher in *ob/ob* mice than in wild-type mice (relative concentrations of 86 versus 21)(Figure 27C). Leptin treatment brought down the lipid to a relative concentration of 11units.

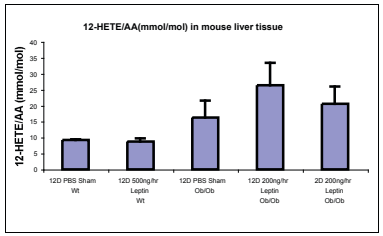
Leptin's Effects on Hepatic Reactive Oxygen Species

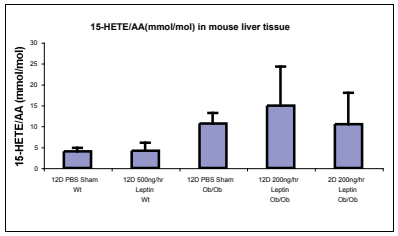
It has been shown that modifications of mitochondrial respiration can cause subsequent changes in reactive oxygen species (ROS) levels, both *in vitro* and *in vivo* (Ambrosio et al., 1993; Chance et al., 1979). Since our study found that leptin treatment of *ob/ob* mice alters mitochondrial substrate oxidation, we wanted to see what, if any, effects this may be having on the formation of ROS. Free radicals *in vivo* react with fatty acids and amino acids to form byproducts that can subsequently be measured to gain a sense of the ROS load in the tissue. To take a deeper look at the effects of leptin on ROS levels, we measured oxidated fatty acid (oxFA) levels from the livers of wild-type + saline, wild-type + leptin, *ob/ob* + saline, *ob/ob* + 2 days leptin, and *ob/ob* + 12 days leptin mice. The results were normalized in two ways and each of them shows a slightly different result. For the sake of thoroughness, I will present both findings in this section.

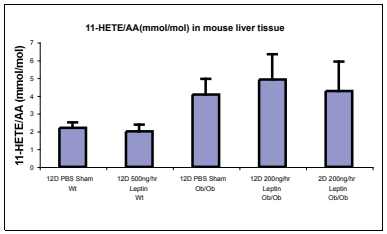
In the first way of normalizing the measurements, the levels of oxFA are expressed as a ratio between the oxFA and its precursor (for example HETE /

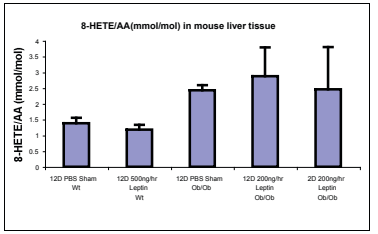
arachidonic acid, or HODE / linoleic acid) This normalization method probably results in the truest measurement of oxidative stress, as increased amounts of precursor will be associated with a proportional increase in the specific oxFA due to mass action. Since we are looking at the ratio of the oxFA and its precursor, differences in starting levels of precursor should be canceled out my employing this method of analysis. When analyzing the results in this method, we find that *ob/ob* mice have higher levels of nearly each of the twelve oxFA measured in liver tissue (Figure 28). Two days of leptin treatment seems to increase these levels slightly, whereas tweleve days of treatment with the hormone seems to increase oxFA levels even more. Though none of the differences observed are statistically significant as they stand, our collaborators believe that they will reach significance with the analysis of more samples.

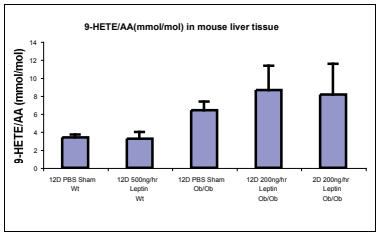
The second method of normalization involves dividing the measured levels of oxFA by the total mg of soluble protein in the liver homogenate. By conducting our analysis in this way we found that *ob/ob* mice had very low levels of the twelve oxFA measured in comparison to wild-type mice (Figure 29). Two days of leptin treatment partially raised oxFAs back to wild-type levels, and twelve days of treatment with the hormone pretty much restored wild-type levels for most of the oxFAs. Again, while none of the differences observed are statistically significant as they stand, our collaborators believe that they will reach significance with the analysis of more samples.

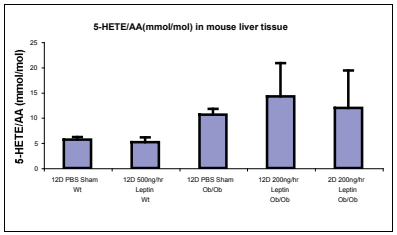


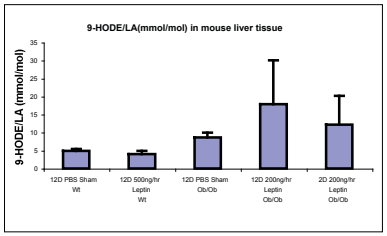


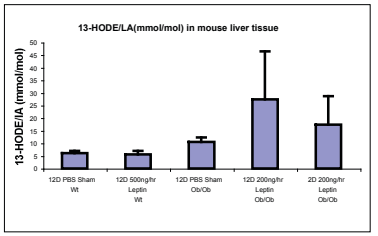


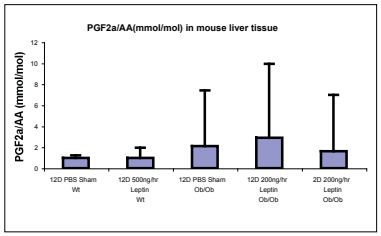












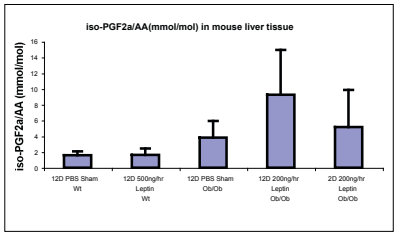
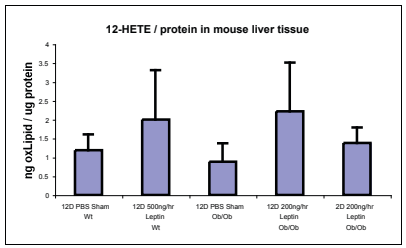
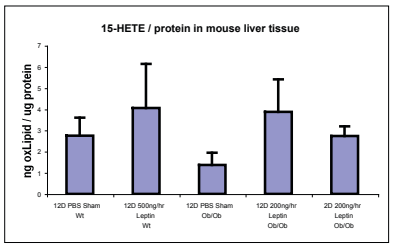
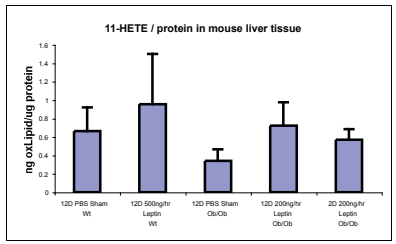
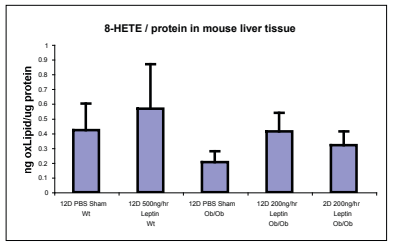


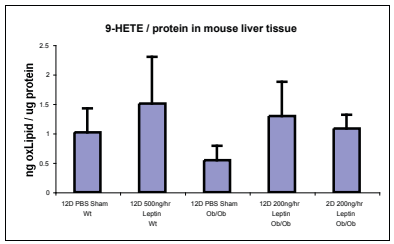
Figure 28. Oxidated Fatty Acids Normalized to their Precursors. Initial trends suggest that ob/ob mice have higher amounts of oxFA/precursor in liver homogenate than wild-type mice. Leptin treatment of these ob/ob mice seems to increase the levels of oxidated fatty acids even further. Though none of the difference are statistically significant as they stand, our collaborators believe that they will reach significance with the analysis of more samples.

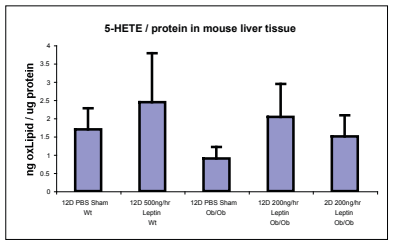


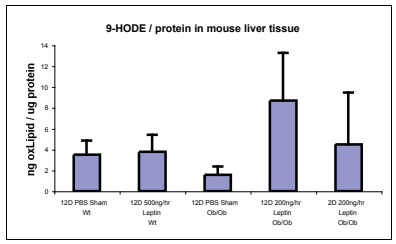


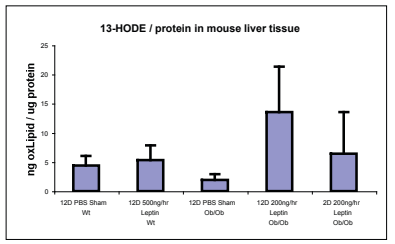


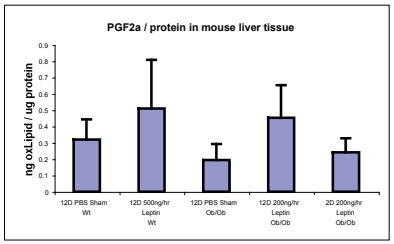












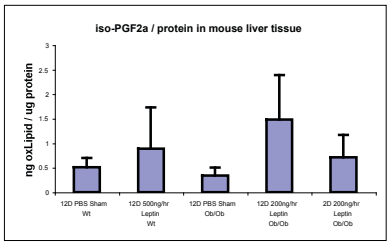


Figure 29. Oxidated Fatty Acids Normalized to the Amount of Protein. Initial trends suggest that ob/ob mice have lower amounts of oxFA/mg soluable protein in liver homogenate than wild-type mice. Leptin treatment of these ob/ob mice restores the levels of oxidated fatty acids back to wild-type levels. Though none of the difference are statistically significant as they stand, ou collaborators believe that they will reach significance with the analysis of more samples.

Leptin Affects Skeletal Muscle Mitochondrial Respiration

Having completed our comprehensive study of leptin's effects on hepatic mitochondria, we wanted to focus on other highly metabolic organs that are good candidates for leptin-mediated changes in energy expenditure. Skeletal muscle is another highly metabolic organ (Rolfe and Brand, 1996; Rolfe et al., 1999), which has been suggested to play a role in the increased energy expenditure associated with leptin treatment. For this reason we set out to measure mitochondrial respiration in this organ, specifically respiratory control ratio (RCR) and its components.

RCR is a ratio that represents coupled respiration divided by uncoupled respiration. Twelve-day leptin treatment of *ob/ob* mice significantly reduces skeletal muscle mitochondrial RCR from 3.41 to 2.65 (Figure 30)($p<0.05$). This change in RCR could be due to a decrease in the amount of coupled respiration, an increase in uncoupled respiration, or both. To explore these possibilities we measured state two, three, four, and FCCP rates next. Mitochondrial extraction from muscle gives a very poor yield, and thus five mice were pooled per sample. Each day we recorded from one sample of *ob/ob* + pbs mice and one sample of *ob/ob* + 12 day leptin treated mice. In order to correct for daily variances in electrode sensitivity - due to freshly made solutions, membrane permeability, electrode deposits, etc - we divided each of the recordings by the total amount of respiration recorded for that day. So ob pbs = ob pbs / (ob pbs + ob lep) ; ob lep = ob lep / (ob pbs + ob lep). This gave us a "normalized ratio" for each recording that we could compare from day to day.

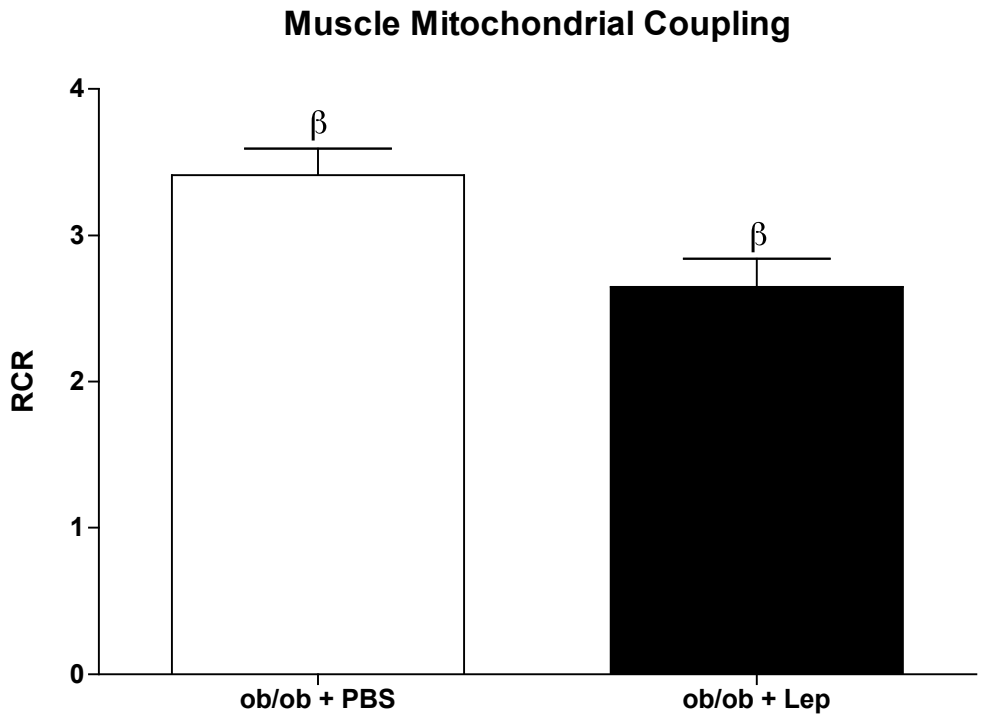


Figure 30. Skeletal muscle mitochondria RCR analysis from ob/ob mice plus/minus leptin. Twelve days of leptin treatment significantly reduces respiratory control ratio (RCR) in the skeletal muscle of ob/ob mice. n=3 pooled samples of 5 mice each, and every recording was done in duplicate. $\beta = p < 0.05$

State two and four rates give insight into mitochondrial leak, i.e. uncoupled respiration, and were both altered by leptin. Twelve-day leptin treatment significantly increased the skeletal muscle state two respiration normalized ratio from 0.45 to 0.55 (Figure 31A)($p<0.01$). This initially indicates that mitochondrial leak is increased in skeletal muscle by leptin, which would explain the decrease in RCR. However, when looking at state four respiration, we find that it is decreased by twelve-day leptin treatment from a normalized ratio of 0.55 to 0.45 (Figure 31B)($p<0.01$). Further analysis regarding the meaning of these rates is carried out in the discussion section.

Having explored uncoupled mitochondrial respiration in skeletal muscle, we next wanted to look at the numerator of RCR, namely coupled respiration. State three and FCCP rates provide a window into this and were thus recorded next. Twelve-days of leptin treatment reduced the skeletal muscle state three normalized ratio from 0.62 to 0.38 (Figure 32A)($p<0.01$). These results indicate that leptin may be decreasing the substrate oxidation system, like it does in liver tissue, and could help explain the changes we saw in RCR. However, though the differences are not yet statistically significant, further replicates may confirm the trend that FCCP rates are also higher with leptin treatment (Figure 32B), which would confound our interpretation of the state 3 rates. The meaning of these results is explored further in the discussion section.

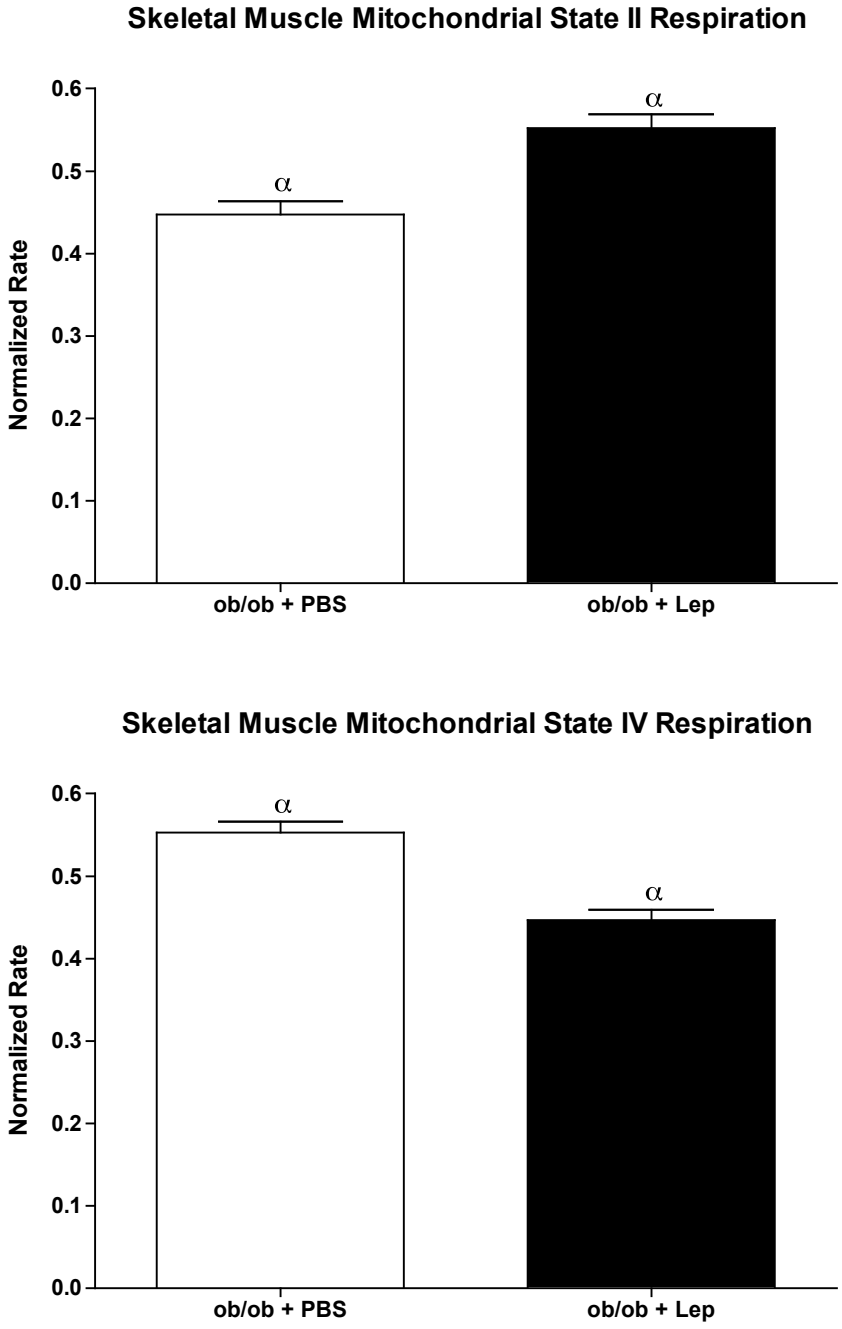
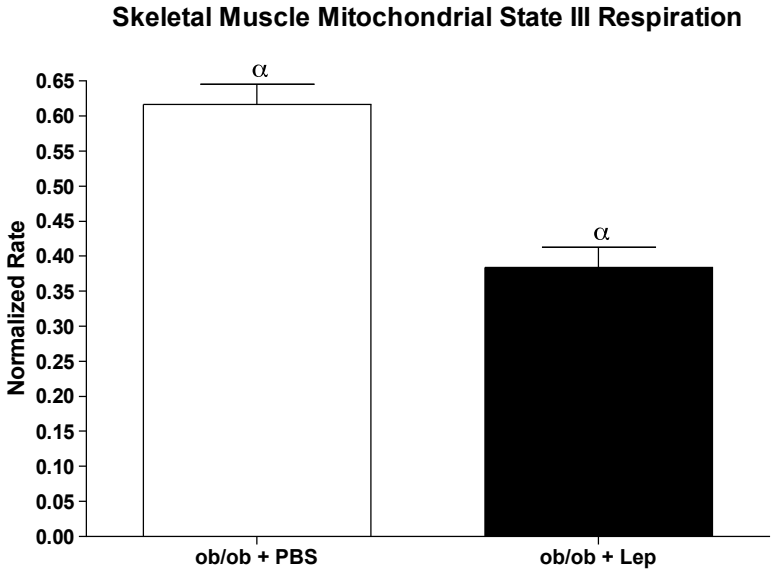


Figure 31. Skeletal muscle leak analysis from ob/ob mice plus/minus leptin. (A) State 2 rates are higher in ob/ob mice treated with leptin, which initially suggested that a higher proton leak in this group may be the cause behind the observed changes in the RCR. (B) However, state 4 rates are lower in the leptin treated group, which make the interpretation of the state 2 rates more complex. n=3 pooled samples of 5 mice each, and every recording was done in duplicate. $\alpha = p < 0.01$



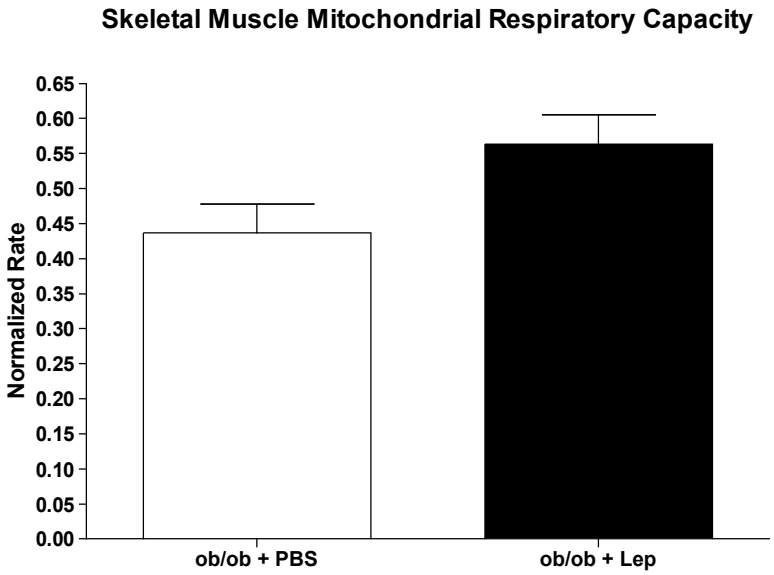


Figure 32. Skeletal muscle substrate oxidation analysis from ob/ob mice plus/minus leptin. (A) State 3 rates are reduced with leptin treatment of ob/ob mice. This suggests that leptin may be decreasing the substrate oxidation system in skeletal muscle, which would be similar to its effects in the liver. (B) However, though the differences are not yet statistically significant, further replicates may confirm the trend that FCCP rates are also higher with leptin treatment, which would confound our interpretation of the state 3 rates. n=3 pooled samples of 5 mice each, and every recording was done in duplicate. $\alpha = p < 0.01$

Leptin Affects Heart Mitochondrial Respiration

Another highly metabolic tissue that makes a good candidate for the site of leptin-mediated increases in energy expenditure is the heart. We therefore set out to measure mitochondrial respiration in this organ, again focusing on RCR and its components. Twelve-day leptin treatment of *ob/ob* mice lowers RCR in heart muscle from 3.02 to 2.08 (Figure 33)($p<0.05$). Again, this change could be due to a reduction in coupled respiration, an increase in uncoupled respiration, or both. To gain insight into which of these possibilities is occurring *in vivo*, we measured state two, three, four, and FCCP rates next. Mitochondrial extraction from the heart, though not as tricky as that from skeletal muscle, also gives a very poor yield, as there is not much heart tissue in each animal. Thus we again pooled five mice per sample, and recorded from one group of *ob/ob* + pbs mice and one group of *ob/ob* + 12 day leptin treated mice per day. In order to correct for daily variances in electrode sensitivity, we again divided each of the recordings by the total amount of respiration recorded for that day, giving us a "normalized ratio" for each recording.

To look at the uncoupled respiration component of RCR, we recorded state two and four rates next. Twelve-day leptin treatment significantly decreased heart mitochondrial state two normalized ratio from 0.57 to 0.43 (Figure 34A)($p<0.05$). State four respiration rates, though not statistically different with the number of replicated performed, also show a trend to decrease with leptin treatment (Figure 34B). Further interpretation of these rates is carried out in the discussion section.

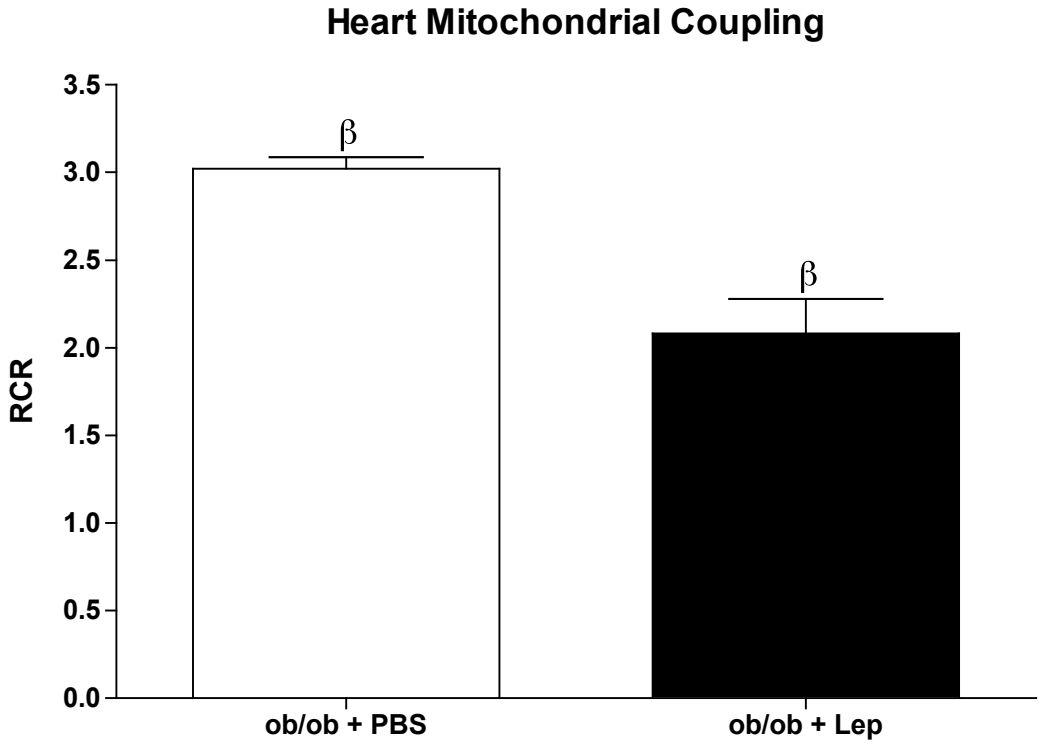


Figure 33. Heart mitochondria RCR analysis from ob/ob mice plus/minus leptin. Twelve days of leptin treatment significantly reduces respiratory control ratio (RCR) in the heart muscle of ob/ob mice. n=3 pooled samples of 5 mice each, and every recording was done in duplicate. $\beta = p < 0.05$

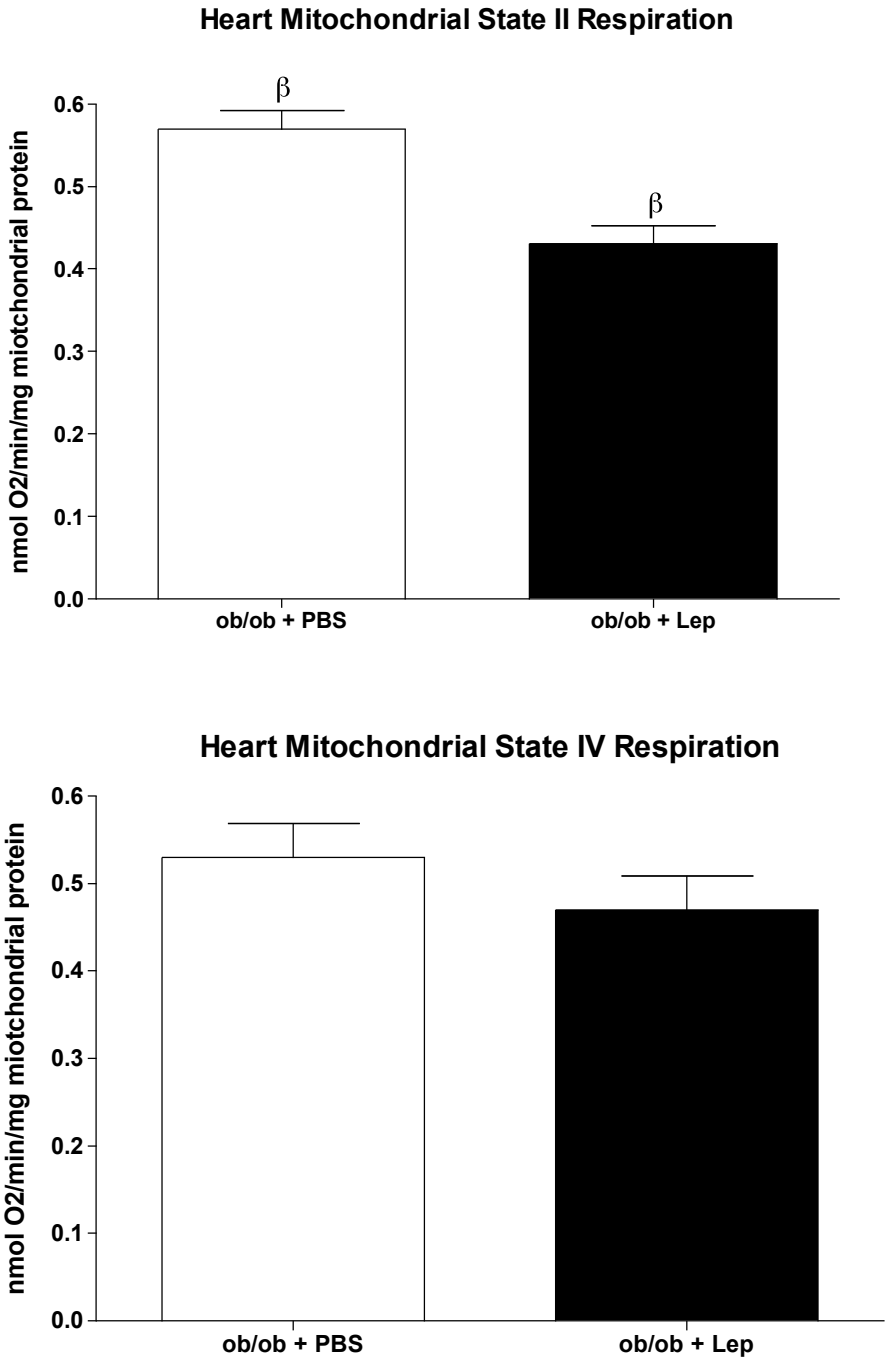


Figure 34. Heart leak analysis from ob/ob mice plus/minus leptin. (A) State 2 rates are lower in ob/ob mice treated with leptin, indicating that proton leak may be lower in the hearts of mice receiving the hormone. (B) State 4 rates are unchanged with leptin treatment. n=3 pooled samples of 5 mice each, and every recording was done in duplicate. $\beta = p < 0.05$

Having explored uncoupled mitochondrial respiration in heart muscle, we next wanted to look at the coupled respiration component of RCR. Twelve-days of leptin treatment reduced the state three normalized ratio in heart mitochondria from 0.60 to 0.40 (Figure 35A)($p<0.01$). These results suggest that leptin may be decreasing the substrate oxidation system, as it does in liver tissue, and could help explain the changes seen in RCR. No difference was seen in FCCP rates between leptin treated and saline treated *ob/ob* mice (Figure 35B).

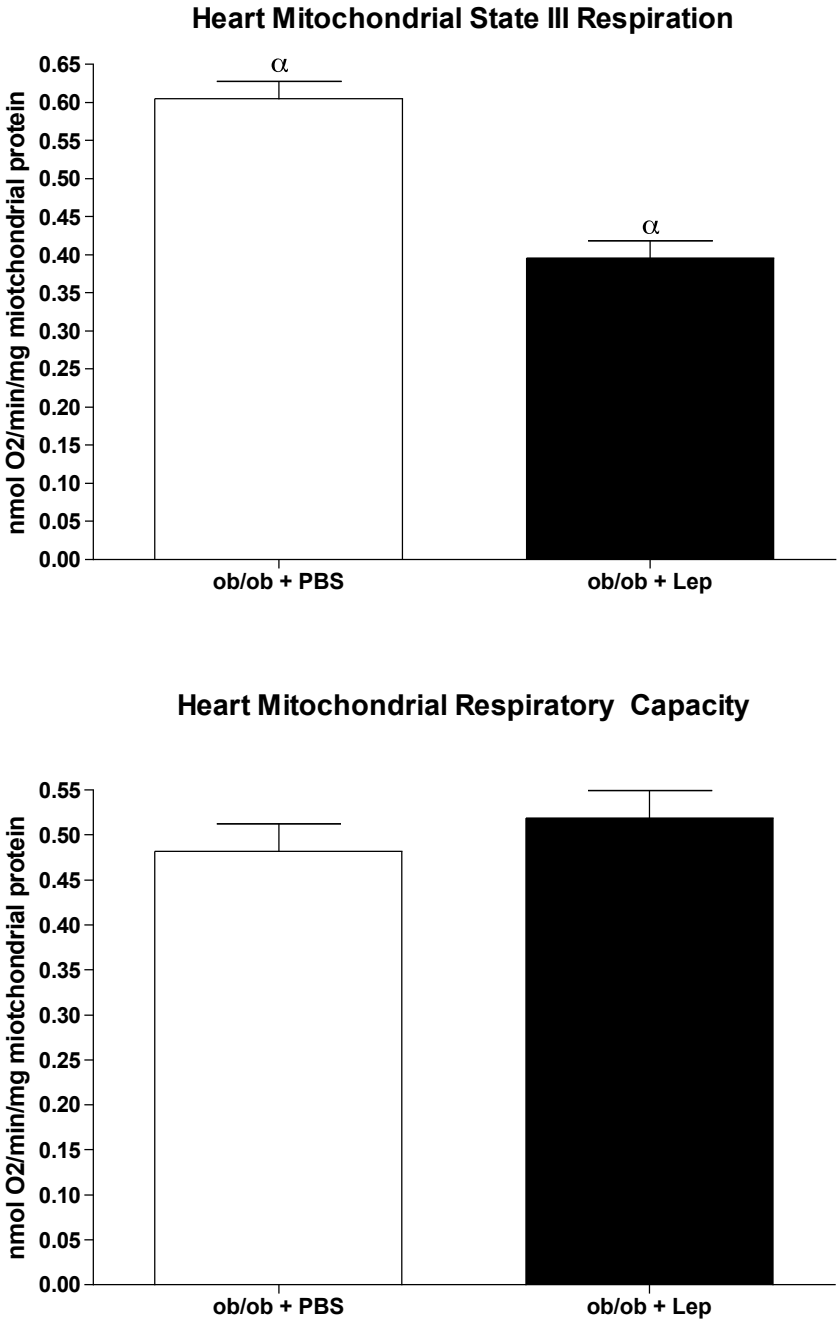


Figure 35. Heart substrate oxidation analysis from ob/ob mice plus/minus leptin. (A) State 3 rates in heart mitochondria are reduced with leptin treatment of ob/ob mice. This suggests that leptin may be decreasing the substrate oxidation system in heart muscle, which would be similar to its effects in the liver. (B) FCCP rates are unchanged with leptin treatment. n=3 pooled samples of 5 mice each, and every recording was done in duplicate. $\alpha = p < 0.01$

Chapter 4: Discussion

Quantitation of Leptin's Effects on Thermogenesis and Metabolism

Body weight homeostasis is a fine balance between energy intake and expenditure (Spiegelman and Flier, 2001), which is innately controlled with an incredible amount of accuracy (Weigle, 1994). Obesity can only occur in the setting where energy intake exceeds energy expenditure, i.e. a state of positive energy balance, for a prolonged period of time. Any intervention that can tip the scale away from this positive energy balance, through either a reduction in caloric intake or an increase in energy expenditure, may be useful in combating obesity.

While research on the treatment of obesity has focused heavily on the importance of food intake, several lines of evidence indicate that alterations of energy expenditure have a powerful, and perhaps dominant, effect on body weight (Friedman, 2004). Indeed, energy expenditure, basal metabolic rates and/or adaptive thermogenesis, are important variables that contribute to human obesity (Bouchard et al., 1990; Levine et al., 1999; Ravussin, 1995). For example, low energy expenditure is highly predictive of future weight gain (Ravussin et al., 1988b; Roberts et al., 1988). Furthermore, weight loss in humans is met with a compensatory decrease in metabolic rate and increase in appetite, which work in concert to resist changes in weight (Leibel et al., 1995). These findings suggest that an understanding of the mechanisms behind changes in energy expenditure is key in combating obesity. Once such a foundation of knowledge has been established, we can work towards

pharmacological agents that mimic and augment the increase in resting metabolic rate induced by exercise. Such agents could be employed to counteract the compensatory metabolic decreases in response to weight loss discussed above, and may be valuable tools in our battle against morbid obesity (Friedman, 2004; Harper et al., 2001a; Lowell and Spiegelman, 2000).

The ob gene product, leptin, is an adipocyte secreted negative feedback hormone that acts on the hypothalamus (Zhang et al., 1994). This hormone, a key regulator in body weight homeostasis, is known to decrease energy intake and increase energy expenditure to tip the scale towards negative energy balance (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). *Ob/ob* knockout mice lacking the hormone leptin have markedly reduced levels of energy expenditure, and become obese even when pair fed to littermate controls (Flier, 2004b; Halaas et al., 1995). This and other studies have indicated that leptin's effects on caloric intake in treated ob mice accounts for only half of the subsequent weight loss that is observed (Campfield et al., 1995; Collins et al., 1996; Halaas et al., 1995; Levin et al., 1996). This is because in addition to reducing caloric intake, leptin infusion of *ob/ob* mice increases total energy expenditure, selectively promotes fat metabolism, and prevents the fall in adaptive thermogenesis that normally occurs with reduced caloric intake (Doring et al., 1998; Halaas et al., 1997; Hwa et al., 1997; Kamohara et al., 1997; Mistry et al., 1997; Pelleymounter et al., 1995; Scarpace et al., 1997; van Dijk et al., 1999).

After the ingestion of a meal, there is typically an adaptive thermogenesis response that tries to counteract any excess calories ingested. By adjusting for changes in physical activity, it has been shown that leptin infusion doubles the amount of feeding related adaptive thermogenesis by acting on both the amplitude and duration of it, and decreases postprandial RQ (Ruffin and Nicolaidis, 2000). This conclusion is consistent with the finding that stimulation of the ventromedial hypothalamus (VMH), a brain center through which leptin exerts some of its catabolic effects, increases metabolism and decreases RQ (Ruffin and Nicolaidis, 1999), while lesions in this area cause obesity (Elmquist et al., 1999a). Therefore, a significant portion of leptin-mediated weight loss can be attributed to an increase in energy expenditure, some of which, but not all, being attributable to adaptive thermogenesis.

Much progress has been made in understanding leptin's role in inducing satiety (Emond et al., 1999; Schwartz et al., 2000a) and the neural networks through which it triggers a rise in energy expenditure (Bachman et al., 2002; Elias et al., 1998). However, little remains known about the downstream effector mechanisms by which the hormone increases energy expenditure, specifically adaptive thermogenesis (Spiegelman and Flier, 2001). In this report we tease apart the mechanisms through which leptin causes weight loss, demonstrate its effects on the mitochondrial structure and metabolism of three energy demanding organs, and delve into the underlying

kinetic and proteomic modifications to develop a deeper understanding of the observed changes.

Our results demonstrate that leptin-mediated weight loss is attributable to three major changes brought about by the hormone, namely its induction of satiety (Figure 9B, arrow 1), its correction of body temperature (arrow 2), and its activation of increases in energy expenditure and basal metabolic rate (arrow 3). The mechanisms underlying the anorexigenic effects of leptin were discussed earlier, and its correction of body temperature may be due to the hormone's stimulation of sympathetic activity in brown fat (Collins et al., 1996; Haynes et al., 1997) and increase of UCP-1 mRNA and protein levels (Cusin et al., 1998; Satoh, 1998; Scarpace et al., 1997). Thus, for the remainder of our investigation we chose to explore the least understood of the changes brought about by leptin, i.e. the increase in energy expenditure and basal metabolic rate.

Lepin-Mediated Changes in Liver Metabolism

The liver is a major player in lipid homeostasis, and comprises a significant fraction of standard metabolic rate (Porter and Brand, 1993). It is also a known contributor to the catabolic effects of leptin (Cohen et al., 2002), and thus makes a good candidate for leptin-mediated increases in metabolic rate and adaptive thermogenesis. Our study into the livers of leptin deficient *ob/ob* mice showed that they are extremely steatotic, a finding that is corrected by twelve-day leptin treatment of the animals. Measurements of triglyceride levels in the livers of animals housed at

22°C or 30°C were able to show that the correction of steatosis by leptin is due to both normalization of body temperature by the hormone, and leptin-induced increases in energy expenditure and basal metabolic rate (Figure 10). These data suggest that part of the weight loss associated with leptin treatment is occurring in the liver. To study the leptin-induced increase in energy expenditure and basal metabolic rate in further detail, we wanted to take a closer look at hepatic metabolism during leptin treatment.

Whole cell oxygen consumption recordings from freshly isolated primary hepatocytes using a Clarke electrode can be useful in identifying changes in the metabolic state of the liver (Brand et al., 1993; Harper and Brand, 1993; Porter and Brand, 1995; Porter, 2001; Schumacker et al., 1993), and were thus employed in our study. Significant differences were found in the basal metabolic rate, uncoupled respiration (i.e. leak), and non-mitochondrial respiration of primary hepatocytes isolated from saline treated and leptin treated *ob/ob* mice (Figure 11). However, contrary to what we were expecting, isolated primary hepatocytes from 12-day leptin infused mice had lower basal metabolic rates and non-mitochondrial respiration than those from saline infused mice (Figure 11A and C). The respiratory leak in 12-day leptin infused *ob/ob* mice was lower than of saline treated mice (Figure 11B), which is consistent with what has been reported previously (Melia et al., 1999). These data show that while the liver plays a definitive role in the change in energy expenditure and basal metabolic rate brought about by leptin, it may not be an effector organ for

the increase in adaptive thermogenesis as we originally thought. To study these leptin-induced changes in hepatocyte energy expenditure in further detail, we went on to look at liver mitochondrial metabolism.

Measurement of the components of mitochondrial respiration has proven to be useful in identifying the changes underlying differences in metabolic rates (Brand et al., 1999; Brand et al., 1994; Brown et al., 1990; St-Pierre et al., 2003). Modular kinetic analysis conducted alongside such measurements allows one to gain further insight into the kinetic properties mitochondrial leak, substrate oxidation, and the phosphorylation system (Brand et al., 1993; Hafner et al., 1990; Porter and Brand, 1995). To further investigate the possibility of mitochondrial metabolic changes in the liver brought about by leptin, we both measured the different components of hepatic mitochondrial respiration, and conducted modular kinetic analysis.

Our results demonstrate that leptin increases the respiratory control ratio (RCR) of liver mitochondria in *ob/ob* mice (Figure 12). This indicates that hepatic mitochondrial respiration is more coupled in mice receiving leptin, a phenomenon that is the opposite of what we expected to see. State two and four rates decrease with leptin treatment as well (Figure 13), which one may interpret as an indication that mitochondrial leak is decreasing. If this were the case, the decreased leak with leptin treatment would explain the increased RCR, as the leak rate is the denominator of the RCR ratio. However, we find that FCCP rates also decrease with leptin treatment (Figure 14), which indicates that the hormone is suppressing the substrate oxidation

system. A decrease in substrate oxidation would cause a subsequent decrease in the proton gradient across the inner mitochondrial membrane, which would reduce the electro-chemical driving force for proton leak. Similarly, a decrease in proton leak would cause a subsequent increase in the proton gradient across the inner mitochondrial membrane, which would increase the resistance against which the substrate oxidation system has to pump protons. These findings raise the proverbial "chicken or egg" question, i.e. is the decreased proton leak leading to a decrease in the substrate oxidation system, or vice versa. To settle this issue we turned to modular kinetic analysis, which measures both leak and substrate oxidation as a function of mitochondrial inner membrane potential, and thus allows us to measure each rate without the confounding effects of the other.

In modular kinetic analysis, one dissipates the mitochondrial inner membrane potential in a stepwise fashion and concurrently uses different assortments of mitochondrial inhibitors to measure the kinetics of the substrate oxidation system, phosphorylation system, and proton leak (Brand et al., 1993; Hafner et al., 1990; Porter and Brand, 1995). Conducting these measurements in *ob/ob* and wild-type mice treated with both leptin and pbs revealed that the leptin-mediated changes in liver mitochondrial metabolism are due to the hormones effect on the substrate oxidation system (Figure 17). Leak and phosphorylation system kinetics were not altered by lepin (Figure 18), and the changes in leak rates seen earlier were secondary to the effects of the substrate oxidation system on the mitochondrial inner membrane

potential. The reason differences in leak rates emerged is because leak in *ob/ob* + pbs mice is more sensitive to changes in inner membrane potential than it is in *ob/ob* + lep, wt + pbs, or wt + lep mice. This can been seen on in figure 18A, where at higher membrane potentials, the leak increases much more in the *ob/ob* + pbs mice than it does in any of the other groups.

In light of these observations, one wonders if the observed change in substrate oxidation kinetics is a secondary response to the hepatic steatosis exhibited by *ob/ob* mice, attempting to increase oxidative phosphorylation rates in the face of compromised fatty acid oxidation. Induction of hypo-leptinemia in wt mice is an innovative way to distinguish between the direct effects of leptin, and those that are secondary to its correction of hepatic steatosis (Montez et al., 2005). Employing this method, we show that state two and four respiration rates aren't affected in hypoleptinemic wt mice (Figure 20). This suggests that leptin levels don't influence mitochondrial leak, a finding that is consistent with what was seen in *ob/ob* mice using modular kinetic analysis. Furthermore, FCCP rates are higher in hypoleptinemic mice (Figre 21), indicating that leptin levels affect the substrate oxidation system, which is also consistent with findings in *ob/ob* mice. Since the metabolic changes during leptin deficiency and restoration are the same in the presence or absence of hepatic steatosis, we can conclude that leptin's control over the hepatic mitochondrial substrate oxidation system is independent of its effects on hepatic fat stores.

Leptin's Influence Over Hepatic Mitochondrial Structure

There is a positive correlation between the metabolic activity of a tissue and the morphologic features of the mitochondria in that tissue - such as size, volume density, and cristae structure (Ghadially, 1988). Furthermore, studies of adaptive thermogenesis have shown oxygen consumption to be correlated with cristae length and density (Martin et al., 1999; Rodriguez-Cuenca et al., 2002). Having observed changes in liver mitochondrial metabolism with leptin treatment, we wanted to measure mitochondria morphology to see if it was affected as well.

Our electron micrographic investigation of *ob/ob* livers reveals that leptin treatment of these mice causes a change in mitochondrial appearance and cristae structure (Figures 22-24). Though leptin does not change individual mitochondrion size, it significantly decreases the numerical and volume density of hepatic mitochondria in *ob/ob* mice (Figure 25). We also know from modular kinetic analysis that the mitochondria from leptin treated *ob/ob* mice have a suppressed substrate oxidation system. This reduction in mitochondrial volume alongside the suppression of the substrate oxidation system, could explain the previously discussed decrease in hepatocyte basal metabolic rate brought about by the hormone. Therefore, the structural data give an indication that both volumetric and metabolic differences in these hepatic mitochondria may be contributing to the changes observed in the liver.

Leptin's Influence Over the Hepatic Mitochondrial Mileu

Leptin has an extensive range of effects that take shape through its influence over numerous molecular pathways (Flier, 2004b; Friedman, 1998). To circumvent the difficulty in identifying which of these pathways exert leptin's influence over substrate oxidation, we purified mitochondria and measured their protein content in *ob/ob* and wild-type mice after 12 days of treatment with either leptin or saline. Interestingly, we found that three components of the electron transport chain were significantly increased in abundance during leptin deficiency, and decreased during leptin infusion (Figure 26). These were cytochrome c oxidase subunit IV, cytochrome c oxidase subunit VIa, and mitochondrial NADH dehydrogenase. Leptin's influence over the protein levels of these mitochondrial respiratory chain components may be the mechanism by which it causes the observed differences in substrate oxidation described earlier.

Also interesting to note were the changes in protein levels of the long chain fatty acid elongase ELOVL5, which were drastically increased in abundance in *ob/ob* mice as compared to wild-type littermates, and were significantly decreased with leptin treatment of these *ob/obs* (Figure 26). The induction of this enzyme has been shown to cause massive hepatic accumulation of mono-unsaturated fatty acids (Wang et al., 2006). Perhaps the low level of this enzyme in leptin deficiency is underlying the hepatic steatosis observed in *ob/ob* animals, and its increase with leptin infusion leads to the correction of this state.

Changes in mitochondrial lipid levels can affect mitochondrial function, and more importantly changes in mitochondrial metabolism can influence the lipid composition of the organelle (Ball and Joel, 1962; Paradies et al., 1999; Paradies and Ruggiero, 1991; Senault et al., 1990). Furthermore, in both diabetes and insulin treatment of the disorder, there are changes induced in the lipid/phospholipid composition of mitochondria (Patel and Katyare, 2006). Having found that leptin affects the mitochondrial substrate oxidation system in a way to decrease mitochondrial metabolism, we wanted to see if we could identify corresponding changes in mitochondrial lipids induced by the hormone. Using mass spectrometry, we were able to show that cardiolipin levels are significantly higher in *ob/ob* mice than in wild-type mice, a difference that was diminished by leptin treatment (Figure 27). Interestingly, cardiolipin interacts with the mitochondrial respiratory chain and can change the efficiency of oxidative phosphorylation (Gohil et al., 2004; Hatch and McClarty, 1998; Hoch, 1992; Hoch, 1998; Koshkin and Greenberg, 2000). Perhaps changes in cardiolipin are relevant to the lepin-induced changes in mitochondrial metabolism.

In addition to affecting the lipid composition of mitochondria, changes in mitochondrial metabolism can influence free-radical production in the organelle (Andrews et al., 2005). Studies have documented increased oxidative stress in obese individuals and have noted its impact on the metabolic syndrome (Furukawa et al., 2004; Keaney et al., 2003). Indeed, many studies argue that oxidative stress and the

signaling pathways it activates are mediators of insulin resistence and beta cell dysfunction in type 2 diabetes (Evans et al., 2002; Evans et al., 2003; Kelley et al., 2002; Schrauwen and Hesselink, 2004). Some go on to link central obesity as a cause of the oxidative stress that subsequently leads to atherosclerosis, endothelial dysfunction, and beta cell failure (Bakker et al., 2000; Perticone et al., 2001).

The mitochondrial electron transport chain is the main producer of reactive oxygen species (ROS) in most cells (St-Pierre et al., 2006). Since we found leptin to have metabolic, proteomic, and lipidomic effects on the electron transport chain of hepatocytes, we wanted to see if this caused a subsequent change in ROS production. To take a deeper look at the effects of leptin on ROS levels, we measured oxidated fatty acid (oxFA) levels from the livers of wild-type + saline, wild-type + leptin, *ob/ob* + saline, *ob/ob* + 2 days leptin, and *ob/ob* + 12 days leptin mice. Our studies found a trend of increased oxFA with leptin treatment (Figures 28 and 29). While these trends are not statistically significant as they stand, our collaborator recording the oxFA levels is confident that they will be with further replicates. Such data would fit in well with previous findings that leptin induces oxidative stress in a variety of organisms (Beltowski et al., 2003; Bouloumie et al., 1999; Yamagishi et al., 2001).

Lepin's Effects on Skeletal Muscle and Heart Metabolism

Calorimetric studies have shown leptin to increase whole body oxygen consumption in *ob/ob* mice (Campfield et al., 1995; Halaas et al., 1997; Halaas et al., 1995; Pelleymounter et al., 1995). However, our measurements show that the

hormone decreases basal metabolic rate in hepatocytes and inhibits the substrate oxidation system in hepatic mitochondria. In order for both observations to be correct, there must be other organs in which leptin increases energy expenditure. The heart and skeletal muscle comprise a significant fraction of standard metabolic rate (Rolfe and Brand, 1996; Rolfe et al., 1999), and thus make good candidates for other tissues upon which leptin may be exerting its metabolic effects. To measure the effects of leptin on these tissues we measured the different components of mitochondrial respiration from *ob/ob* mice treated with either leptin or saline for twelve days.

Our results show that, opposite to its effects in the liver, leptin significantly decreases respiratory control ratio (RCR) in skeletal muscle (Figure 30). To explore the cause(s) behind this change, we took a further look at mitochondrial leak and coupled respiration, the two components of RCR. State two rates were found to be higher with leptin treatment (Figure 31A), which initially indicated that mitochondrial leak might be increased in animals receiving the hormone. This could help explain the lower RCR, as mitochondrial leak is the denominator in the RCR ratio. However, we also observed that state four respiration is decreased with leptin treatment (Figure 31B), which would suggest that mitochondrial leak is decreased. The conflicting state two and four findings confound our analysis of uncoupled respiration and do not allow us to come up with a definitive conclusion regarding changes to mitochondrial leak. To resolve this issue, one would have to do modular kinetic analysis using a TPMP electrode.

A similar situation arises with state three and FCCP rates in skeletal muscle mitochondria. Leptin treatment significantly lowers state three rates (Figure 32A), which indicates that the hormone may decrease coupled respiration. This would also explain the changes to RCR, as coupled respiration is the numerator in the RCR ratio. However, though they are not statistically significant as they stand, FCCP rates show a nearly significant trend of increasing with leptin treatment (Figure 32B). Perhaps further replicates of this recording would bring the trend to significance, in which case there would be a conflict regarding the interpretation of coupled respiration. Once again, modular kinetic analysis using a TPMP electrode could be employed to resolve the issue.

Turning our attention to heart mitochondria, we find that twelve days of leptin treatment also decreases the RCR in this tissue (Figure 33). To explore the cause(s) behind this change, we again took a further look at mitochondrial leak and coupled respiration. State two and four rates are reduced with leptin treatment (Figure 34), which indicates that mitochondrial leak may be decreased in the hearts of mice receiving the hormone. At the same time state three rates are decreased with leptin treatment as well (Figure 35), which suggests that substrate oxidation may be reduced in the hearts of leptin treated *ob/ob* animals. This raises the proverbial chicken and egg question once more. Is the decreased leak causing a buildup of the proton gradient, and thus slowing substrate oxidation rates by forcing the mitochondrial respiratory chain to pump against a higher eletro-checmical force? On the other hand,

are decreased substrate oxidation rates resulting in a smaller mitochondrial inner membrane proton gradient, causing subsequent decreases in proton leak rates? To answer these questions, one could conduct modular kinetic analysis on isolated heart mitochondria.

Conclusion

The primary goal of the work described here was to gain a better understanding of the cellular and molecular mechanisms underlying leptin's metabolic effects. The experiments in this thesis start off by identifying the three different ways in which leptin causes weight loss, namely the induction of satiety, the normalization of body temperature, and the increase of energy expenditure and basal metabolic rate. They then go on to take an in depth look at the liver, skeletal muscle and heart, which we felt were three strong candidate organs for involvement in the leptin mediated increase of energy expenditure.

We report that leptin treatment of *ob/ob* mice reduces the basal metabolic rate, uncoupled respiration (i.e. leak), and non-mitochondrial respiration of primary hepatocytes. By recording the components of mitochondrial respiration and conducting modular kinetic analysis, we show that leptin treatment of *ob/ob* mice decreases the substrate oxidation system in the liver. In vivo withdrawal studies show that these changes are directly caused by leptin, and are not downstream of the hormone's correction of hepatic steatosis. Furthermore, electron microscopy and morphometry studies reveal that in addition to causing metabolic changes in mitochondria, leptin treatment changes mitochondrial structure, size, and volume density in the liver. Thus we are able to provide both a metabolic and structural basis for our observed effects of leptin on hepatocytes.

We next wanted to see if we could find the proteomic and lipidomic changes underlying the metabolic and structural changes discussed above. Proteomic analysis using iTRAQ tandem mass spectrometry quantification revealed that leptin-mediated changes in three components of the mitochondrial respiratory chain could account for the aforementioned metabolic effects of leptin in the liver. It also showed that drastic changes in levels of the enzyme ELOVL5 might be responsible for the hepatic steatosis exhibited with leptin deficiency, and for its correction with leptin treatment. Furthermore, mass spectrometry showed differences in mitochondrial cardiolipin levels that are associated with the observed leptin-mediated changes in the liver. Finally, our measurements of oxidated fatty acids show a trend that leptin may be altering the levels of reactive oxygen species in hepatic mitochondria. Thus we are able to provide a proteomic and lipidomic basis for our observed effects of leptin on liver mitochondrial metabolism and hepatic steatosis, and show additional effects of the hormone on the mitochondrial milieu.

Having completed a thorough investigation into leptin-mediated changes in the liver, we moved on to the other two candidate organs, the heart and skeletal muscle. Our findings show that the respiratory control ratio (RCR) is reduced in mitochondria isolated from both the heart and skeletal muscle of mice treated with leptin for 12 days. In skeletal muscle, state two and four recordings are somewhat conflicting about leptin-mediated changes in mitochondrial leak, and state three and FCCP rates are somewhat conflicting about changes in coupled respiration. The

mitochondrial rates paint a clearer picture in the heart, showing that both leak and coupled respiration are decreased with leptin treatment. However, it is likely that there is interplay between these rates, and one cannot say which is influencing the other. To resolve both this issue and the conflicts in interpretation of skeletal muscle rates, one needs to conduct modular kinetic analysis. Thus, our results show that leptin clearly modulates mitochondrial respiration in the heart and skeletal muscle, though we cannot yet say exactly how it does so.

In conclusion, our analysis of the liver shows that leptin decreases mitochondrial substrate oxidation and volume density, leading to a resultant decrease in hepatocyte basal metabolic rate. It does so by changing levels of three proteins in the mitochondrial respiratory chain. These effects are a direct result of leptin and not secondary to its correction of hepatic steatosis, which is likely occurring through its influence on the fatty acid elongase ELOVL5. We also clearly show that skeletal muscle and heart metabolism are changed with leptin treatment, and lay the foundation for future work on these tissues. These observations advance our understanding of the catabolic effects of leptin, and bring us one step closer to coming up with potential therapeutic treatments for obesity that approach the problem from a completely new angle.

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