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Leptin and the Control of Body Weight and Metabolism: Role for Stearoyl CoA Desaturase-1 and Other Leptin-Regulated Genes in the Liver

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Leptin and the Control of Body Weight and Metabolism: Role for Stearoyl CoA Desaturase-1 and Other Leptin-regulated Genes in the Liver

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Dedication

This thesis is dedicated to the people in my life without whom this work would not have been possible. To my parents for always putting their childrens' interests before their own. Their countless sacrifices, strong values, dedication to education, and unwavering love and encouragement have made me the person I am. To my brother Justin for his constant support and for his sense of humor which has always kept life in its proper perspective. To Patricia for her love and dedication throughout this entire process. She has shared the trials and tribulations of research, making them tolerable, as well as the successes, making them that much sweeter.

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A. Soukas, N.D. Socci, P. Cohen, A. Viale, and J.M. Friedman. *In preparation.* Acute decreases in plasma leptin activate a novel program of lipid re-accumulation in white adipose tissue.

Abstract

Leptin is an adipocyte-derived hormone that regulates energy balance, metabolism, and the neuroendocrine response to altered nutrition. Mice lacking leptin (*ob/ob*) or its receptor (*db/db*) are massively obese and hyperphagic. They also have increased triglyceride deposits in multiple peripheral organs, including the liver, which is massively enlarged and steatotic. Leptin elicits a metabolic response that cannot be explained by its anorectic effects alone. This response is characterized by weight loss, the depletion of lipid from peripheral tissues, and enhanced insulin sensitivity. The mechanisms underlying these actions were largely unknown. This thesis addressed leptin's metabolic effects on the liver, an organ with a critical role in lipid metabolism and glucose homeostasis.

The relative contribution of central vs. peripheral leptin action on body weight, neuroendocrine function, and lipid metabolism had not been tested genetically. To address this question, mice with either neuronal ($ObR^{SynI}KO$) or hepatocyte-specific ($ObR^{Alb}KO$) deletion of the leptin receptor were studied. $ObR^{SynI}KO$ mice were massively obese and had neuroendocrine abnormalities resembling those of *db/db* mice, whereas $ObR^{Alb}KO$ mice displayed normal body weight. $ObR^{Alb}KO$ mice had normal liver triglyceride levels, while $ObR^{SynI}KO$ mice had enlarged fatty livers, indicating that the liver abnormalities of *ob/ob* and *db/db* mice are secondary to defective leptin action in the brain.

Microarrays were used to explore the basis for leptin's unique effects on the liver. Genes with altered expression in *ob/ob* liver were identified, and gene expression was profiled following a time course of weight loss induced by either leptin treatment or food

restriction. Cluster analysis demonstrated a unique transcriptional response to leptin, distinct from pair-feeding. Based on these findings, an algorithm was developed to identify and rank leptin-regulated genes for further functional analysis. The two most strongly leptin-regulated genes were stearoyl-CoA desaturase-1 (SCD-1) and insulin-like growth factor binding protein-2 (IGFBP-2). SCD-1 was specifically repressed during leptin-mediated weight loss, and mice lacking SCD-1 showed markedly reduced adiposity on both a lean (ab^J/ab^J) and *ob/ob* background (ab^J/ab^J; *ob/ob*), despite higher food intake. ab^J/ab^J; *ob/ob* mice also showed a complete correction of the hypometabolic phenotype and hepatic steatosis of *ob/ob* mice, suggesting that fatty acid oxidation is enhanced in the absence of SCD-1. Furthermore, both alcoholic and nonalcoholic fatty liver disease secondary to lipodystrophy were markedly attenuated in SCD-1 deficient mice. IGFBP-2 was specifically induced during leptin treatment. Double mutant *IGFBP-2*$^{-/-}$*;ob/ob* mice were partially resistant to leptin, suggesting that IGFBP-2 induction may be necessary for the full spectrum of leptin's metabolic effects.

Thus, central leptin action coordinates a specific transcriptional response in the liver, which mediates the metabolic effects of leptin. SCD-1 and IGFBP-2 both have a role in leptin-mediated weight loss. Further study of these and other leptin-regulated genes may further elucidate the molecular basis for leptin's unique effects on metabolism.

Chapter 1: Introduction

Obesity as a health problem

Medical and psychological effects of obesity

Obesity is an increasingly urgent medical and public health concern. The effects of obesity and overweight have now permeated society. The diet industry generates billions of dollars of revenue each year, with over 30% of adults in the United States reporting that they are attempting to lose weight (Serdula et al. 1999; Friedman 2000a). Obesity has been estimated to cost citizens over $70 billion annually in both health care costs and lost productivity (Wickelgren 1998). As further evidence of the far-reaching effects of obesity, recent news stories have described a class action lawsuit against fast food chains by a morbidly obese man (Reaves 2002), an expanding market in oversized children's clothing (Erman 2002), and the banning of soda sales by the Los Angeles school board in order to combat childhood obesity (Lota 2002). While these stories can be viewed with a mixture of pessimism and alarm, an examination of the health consequences and incidence of obesity illustrates the staggering scope of this issue.

Obesity is associated with increased morbidity and mortality due to type 2 diabetes mellitus, coronary heart disease, cancer, hypertension, osteoarthritis, cholelithiasis, and sleep apnea (Kopelman 2000). Actuarial studies have shown that mortality and body weight are highly related. When smokers were removed from the analysis, lowest mortality was found in those who are slightly underweight, with mortality rising in parallel with body weight (Lew 1985). Results from the Nurses Health

Study of ~115,000 women showed a direct relationship between body weight and overall mortality, as well as mortality specifically from cardiovascular disease (Figure 1.1) (Manson et al. 1995). In a 26-year follow-up of over 5000 participants in the Framingham Heart Study, obesity, independent of other risk factors, was found to be a predictor of cardiovascular disease (Hubert et al. 1983). Further analysis of this population found that men with body weight even 20% greater than normal showed elevated mortality (Garrison et al. 1983).

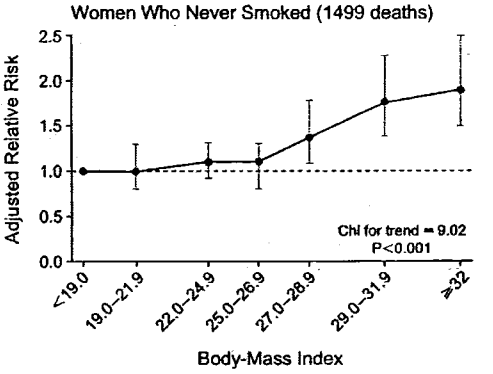


Figure 1.1 Relative risk of death vs. body mass index. Association between relative risk of death and BMI in women who never smoked. Relative risk is directly related to BMI (Taken from Manson *et al.*, 1995).

Obesity not only contributes to illness, but also carries with it a painful social stigma in modern society. Overweight people are more likely to be thought of as unhappy and as possessing negative personality traits (Hiller 1981). Studies have shown that obese people are the victims of discriminatory attitudes and behavior in three major facets of life: education, employment, and health care (Puhl and Brownell 2001). This stigmatization contributes to psychological distress, poor self-esteem, and perhaps worsening obesity (Myers and Rosen 1999).

Defining overweight and obesity

The most widely used metric for assessing overweight and obesity is body mass index (BMI), which is equal to weight in kilograms, divided by the square of height in meters. Other measures for defining obesity include waist circumference, skin fold thickness, and bioimpedance (Kopelman 2000). BMI is highly correlated with body fat,

which is in turn, associated with adverse effects of obesity. When analyzing BMI, however, a few caveats need to be considered. BMI does not distinguish lean mass from fat mass, nor does it describe the distribution of body fat, or take into account differences in body proportions. The World Health Organization classifies a BMI from 18.5-24.9 as normal and healthy. Individuals with BMI from 25.0-29.9, 30.0-39.9, and >40 are respectively classified as overweight, obese, and morbidly obese (Committee 1995; Organization 1997). BMI is closely associated with the incidence of type 2 diabetes, cholelithiasis, coronary heart disease, and hypertension, showing a linear relationship for BMI < 30, but more of an exponential relationship for BMI above 30 (Willett et al. 1999). Data on women from the Nurses' Health Study and on men from the Health Professionals Follow-up Study indicate that individuals with a BMI > 35 have a 20-fold increased risk of developing diabetes, relative to individuals with BMI between 18.5 and 24.9 (Field et al. 2001).

Rising incidence of obesity

By any criteria, the incidence of obesity (BMI > 30) and overweight (BMI 25-29.9) in the United States, other industrialized nations, and even in the developing world is increasing rapidly (Taubes 1998). The most recent data indicate that the prevalence of overweight and obesity is 34% and 27% respectively in American adults (Yanovski and Yanovski 2002). By comparison, according to the National Health and Nutrition Examination Surveys (NHANES), only 12.8% of American adults met the criteria for obesity in 1962 (Kuczmarski et al. 1994). Certain minority populations are affected at much greater levels, with more than 10% of African-American women meeting the cutoff

for morbid obesity (BMI > 40) (Flegal et al. 1998). Similar, but less severe trends have also been observed throughout Europe, where recent estimates cite 15% of men and 22% of women as obese (Project 1988). Among Pacific Islanders, rates of obesity are markedly higher reaching a prevalence of greater than 60% in certain populations (Hodge et al. 1995; Shmulewitz et al. 2001). Rates of obesity have also risen in Japan, Korea, China, and Thailand, countries where obesity has not traditionally been a concern (Popkin 1994).

Obesity is not only affecting adults in greater numbers, but is an increasing concern in children and adolescents as well. Since 1976, the prevalence of overweight, among children and adolescents in the US, has more than doubled (Troiano et al. 1995) (Yanovski and Yanovski 2002). This trend has been linked to a rise in the incidence of Type 2 diabetes and other sequellae of increased body weight in this age group (Sinha et al. 2002). In addition, early infancy, 5-7 years of age, and adolescence are critical periods where the presence of obesity markedly increases the risk for obesity in adult life (Dietz 1994). Thus, obesity not only impacts on health in children, but also predisposes them to a lifetime of health problems.

Causes of obesity

A consequence of environment or biology?

In basic terms, obesity, a disorder of energy homeostasis, develops when energy intake exceeds energy expenditure. One view, popular among the public, is that obesity is the result of a lack of willpower and gluttony, coupled with extreme laziness. In today's consumer-driven society, Americans are bombarded with an array of "super sized," high

calorie fast food. In addition, low fat foods are aggressively, and deceptively marketed as a guilt-free alternative that can be consumed without consequences. The amenities of modern society have not only led to higher caloric intake, but have also facilitated decreased physical activity. Children in the US are spending increasing amounts of leisure time watching television or playing video games, activities that have been postulated to be partly responsible for the rise in childhood obesity (Robinson 2001). Furthermore, low physical activity and reduced energy expenditure have been found to be risk factors for weight gain (Ravussin et al. 1988; Rissanen et al. 1991). The above information supports an environmental basis for obesity, a hypothesis that has been further bolstered by studies of migrant populations. Among Asian Americans, the likelihood of overweight and obesity increases with the number of years spent in the US (Lauderdale and Rathouz 2000). Obesity is also more prevalent in American born than in foreign-born individuals, and increases markedly in second and third generation immigrants (Popkin and Udry 1998).

An alternative theory for the etiology of obesity posits that weight is maintained by a precise physiological mechanism. Weight, whether normal or not, tends to remain stable in most adult humans and other mammals, implying the existence of a precise homeostatic circuit. Most adults gain a small amount of weight over the course of each decade, and calculations indicate that this trend requires that food intake and energy expenditure match one another within 0.17% over this period (Weigle 1994). Furthermore, when food intake is restricted to maintain a 10% reduction in body weight, energy expenditure decreases to offset this perturbation (Leibel et al. 1995). The inherent stability in body weight led to the 'set point' hypothesis, which proposes that food intake

and energy expenditure are coordinately regulated to maintain a relatively constant body weight (Harris 1990; Friedman and Leibel 1992). This hypothesis suggests the presence of afferent signals indicating the level of energy stores, which are sensed and integrated, and then transduced into efferent signals controlling appetite and energy expenditure. Early physiology experiments indicated that the hypothalamus might be the central integrator, where afferent signals are sensed and efferent responses are generated, and that adipose tissue might be the source of the afferent signal (Kennedy 1950; Kennedy 1953). In support of this theory, lesions of the ventromedial hypothalamus lead to hyperphagia and obesity, while lesions of the lateral hypothalamus result in increased energy expenditure, decreased food intake, and weight loss (Hetherington and Ranson 1942; Powley and Keesey 1970).

The above discussion supports the view that body weight is determined by environment and behavior, in conjunction with a precise homeostatic loop. Why then has this physiological system not countered the rise in body weight associated with the sedentary lifestyle and abundance of calories in modern society? According to the "thrifty genotype" hypothesis, in ancient hunter-gatherer societies, where food was scarce and obtaining nutrients required significant energy expenditure, the ability to efficiently store energy in the form of adipose tissue provided an adaptive advantage (Neel 1999). Individuals capable of most efficiently storing energy in adipose tissue would be most likely to survive frequent periods of food deprivation, and in turn, pass these genes on to their offspring. Now, thousands of years later, in an environment where calorically dense foods are plentiful and technology has permitted a sedentary existence, the ready storage of energy as adipose tissue no longer serves a beneficial purpose, and instead contributes

to the obesity epidemic. This hypothesis explains the recent rise in obesity and further illustrates that this disorder is the result of an intricate connection between genes and environment.

A genetic basis for obesity

While body weight can be heavily influenced by environment, obesity has a definite genetic component. Twin studies, adoption studies, and patterns of familial aggregation all support a genetic basis for obesity. An analysis of identical twins either reared together or apart, found that the correlation in BMI was only slightly lower in twins raised apart, than in those raised together (Stunkard et al. 1990). Furthermore, when pairs of identical twins were subjected to periods of positive or negative energy balance, the rate and proportion of weight gain, as well as the site of fat deposition, were more similar within than between pairs (Bouchard et al. 1990). A large study of more than 100,000 individuals from either biologic or adoptive relationships found a BMI correlation of 0.70 in monozygotic twins and 0.32 in dizygotic twins, which corresponds to a heritability of 50-90% (Maes et al. 1997). This heritability coefficient is equivalent to that for height and greater than those for many other traits considered to have genetic underpinnings (Friedman 2000a). In order to reconcile the disproportionate prevalence of overweight and obesity in westernized societies with this convincing genetic data, many researchers have proposed that susceptibility to obesity is under genetic control and that behavior and environment determine the phenotypic expression of these susceptibility genes (Barsh et al. 2000).

Obesity can thus be viewed as a complex, polygenic disease in which genetic modifiers interact with the environment to determine an individual's adiposity. However, rare, Mendelian single gene disorders giving rise to obesity in humans and rodents, have been invaluable in elucidating the physiological basis for body weight regulation. Recessive mutations in the mouse genes *obese (ob)* and *diabetes (db)* lead to an identical syndrome of marked obesity and diabetes, resembling human morbid obesity (Coleman 1978). These mice weigh approximately three times more than normal littermates, with five times more body fat. Parabiosis experiments, in which the circulations of two mice are fused, suggested that the *ob* gene encodes a circulating factor regulating appetite and energy expenditure and that *db* encodes its receptor (Figure 1.2) (Coleman 1978). When the circulations of a wild-type and an *ob/ob* mouse are fused, the *ob/ob* mouse decreases its food intake and loses weight, indicating that a soluble factor exists in wild-type mice, which is absent in *ob/ob* mice (Figure 1.2, bottom panel). This factor also exists in *db/db* mice, as indicated by weight loss in *ob/ob* mice parabiosed to *db/db* mice (Figure 1.2 middle panel). However, *db/db* mice are clearly resistant to this factor and overproduce it,

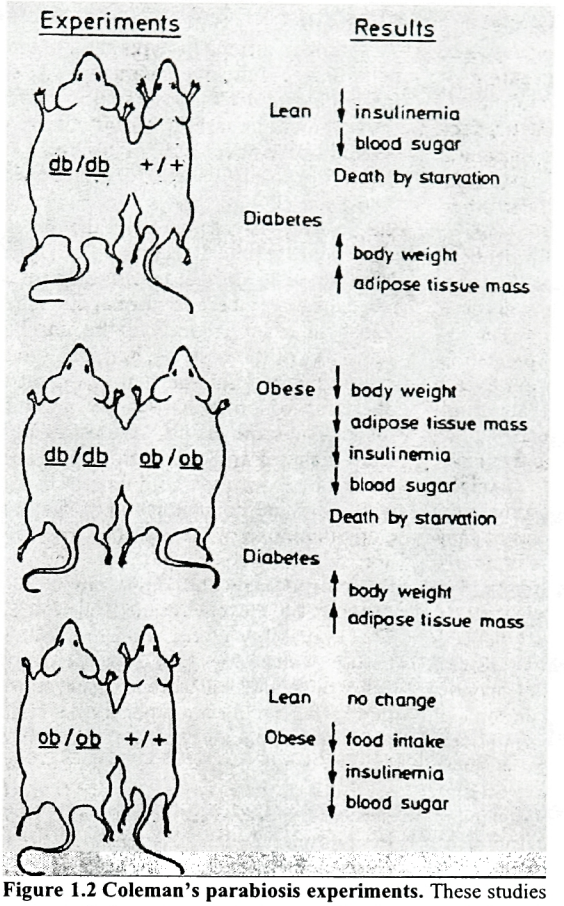


Figure 1.2 Coleman's parabiosis experiments. These studies suggested that *ob* encoded a soluble factor and that *db* encoded its receptor (Taken from Coleman, 1978).

because parabiosis of *db/db* and lean mice has no effect on *db/db* mice, but causes death by starvation in wild-type animals (Figure 1.2, top panel).

A molecular basis for energy homeostasis

Leptin and the regulation of body weight

Coleman's hypothesis was validated with the positional cloning of the *ob* gene, which was shown to encode an adipose tissue-derived, 16 kilodalton circulating hormone, named leptin (Zhang et al. 1994; Friedman and Halaas 1998). Leptin is also expressed at lower levels in the placenta, skeletal muscle, and gastric epithelium (Masuzaki et al. 1997; Bado 1998; Wang 1998). In the ob^{2j} mutant, an Etn transposon is inserted into the first intron of the gene, generating hybrid RNAs, where the splice donor of the non-coding first exon is joined to splice acceptors in the transposon, preventing the synthesis of mature RNA (Moon and Friedman 1997). In the more commonly studied ob^{1j} mutant, a nonsense mutation in the leptin gene leads to translation of a truncated protein that is not secreted into the circulation (Zhang et al. 1994). In this mutant, the levels of *ob* RNA are elevated, suggesting that leptin expression is under feedback control.

The initial data indicated that leptin might be the afferent signal in a negative feedback loop regulating adiposity. Leptin is expressed in all adipose depots, and its expression is increased roughly 20-fold in mice with hypothalamic lesions or in *db/db* mutants (Maffei et al. 1995a). Plasma leptin levels are increased proportionately to body mass in obese mice and humans, and fall in both following weight loss (Maffei et al. 1995b; Considine et al. 1996).

These results suggested that leptin was an indicator of nutritional excess, with high levels of circulating leptin signaling an animal to decrease food intake and increase energy expenditure. Administration of leptin by intraperitoneal injection or subcutaneous infusion leads to a dose-dependent decrease in body weight and food intake in *ob/ob* and wild-type, but not *db/db* mice (Figure 1.3) (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995; Stephens et al. 1995). In both *ob/ob* and wild-type mice, leptin-induced weight loss is specific to adipose tissue, with no affects on lean body mass (Halaas et al. 1995; Pelleymounter et al. 1995). Adenovirus-mediated leptin gene therapy also specifically depletes adipose tissue (Chen et al. 1996a). Furthermore, leptin treatment improves hyperglycemia, hyperinsulinemia, and hypercorticosteronemia in *ob/ob* mice (Halaas et al. 1995; Pelleymounter et al. 1995; Stephens et al. 1995). When wild-type mice are treated with leptin, the reduction in food intake only persists until the adipose tissue has been depleted, after which, food intake returns to normal, without any weight being regained (Halaas et al. 1997). The signal responsible for attenuating leptin's effects in wild-type mice remains unknown.

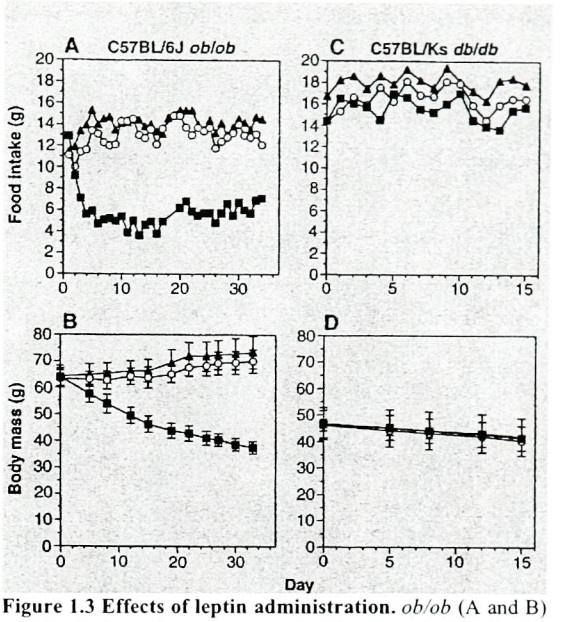


Figure 1.3 Effects of leptin administration. *ob/ob* (A and B) and *db/db* (C and D) mice received daily injections of leptin (5 μg/g per day) (solid squares), daily injections of PBS (open circles), or no treatment (solid triangles) (Taken from Halaas *et al.*, 1995).

An alternate view of leptin's role is as a signal of nutritional deprivation, with low leptin levels initiating an adaptive response to conserve energy, manifested by hyperphagia, decreased energy expenditure, and shutdown of the reproductive and other

endocrine axes. *ob/ob* mice, which lack circulating leptin, are hyperphagic and hypometabolic and exhibit other characteristics of starvation, such as decreased body temperature, impaired immune function, and infertility (Coleman 1978). Concurrent leptin administration largely corrects fasting induced changes in testosterone, luteinizing hormone, thyroxine, corticosterone, ACTH, and estrous (Ahima et al. 1996). Leptin also corrects the starvation induced fall in serum growth hormone and can reverse the immunosuppressive effects of starvation (LaPaglia et al. 1998; Lord 1998). Leptin administration can restore fertility in female *ob/ob* mice (Chehab et al. 1996). Leptin's effects on the reproductive axis are further evidenced by the early onset of puberty in leptin treated mice, and the delayed onset of puberty and cessation of ovulation in extremely thin, hypoleptinemic women (Ahima et al. 1997; Chehab et al. 1997). In addition, leptin injection into the third ventricle of ovariectomzied rats leads to a significant increase in plasma luteinizing hormone (Yu et al. 1997).

The above findings indicate that leptin's role may be to signal nutritional deprivation to the endocrine system. However, leptin is also clearly important in preventing weight gain in periods of nutrient excess. A broader view holds that leptin dynamically regulates appetite, energy expenditure, and neuroendocrine function to maintain body weight within a narrow range.

Leptin signaling

A high-affinity leptin receptor (Ob-R) was identified from mouse choroid plexus by expression cloning (Tartaglia et al. 1995). Ob-R was found to be a membrane spanning receptor, related to the cytokine receptor family. Positional cloning of the

mouse *db* gene proved that it encodes Ob-R, which has five alternatively spliced isoforms, ObR (a-e) (Figure 1.4a) (Chen et al. 1996b; Chua et al. 1996; Lee et al. 1996). Strains of *db/db* and *fa/fa* rats have been characterized and found to contain different receptor defects (Philips et al. 1996; Lee et al. 1997; Li et al. 1998). C57Bl/Ks *db/db* mice contain an insert leading to an alternatively spliced transcript with a premature stop codon, preventing the expression of Ob-Rb, while other isoforms remain intact (Figure 1.4b) (Chen et al. 1996b; Lee et al. 1996). These mice have the same phenotype as other strains of *db/db* mice, lacking all Ob-R isoforms, demonstrating that Ob-Rb is critical for leptin's effects on body weight.

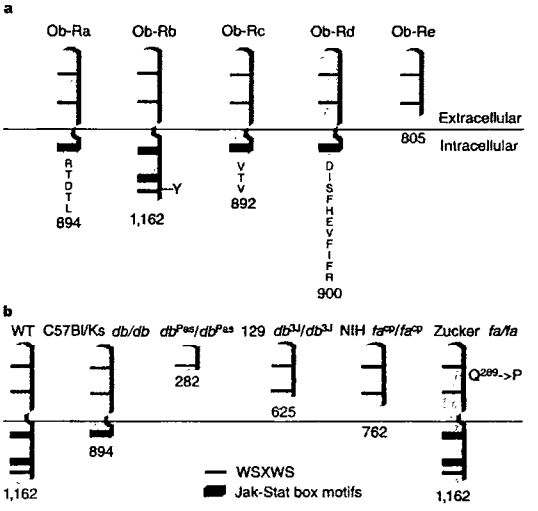


Figure 1.4 Leptin receptor isoforms and receptor mutations in rodent models of obesity (a) The structure of the five ObR splice variants and (b) the nature of different ObR mutations (Taken from Friedman and Halaas. 1998).

Ob-Rb is the only isoform that has a long intracellular domain capable of transducing signals. It signals through a cytoplasmic tail with consensus Jak-Stat binding motifs and three tyrosine residues that are targets for phosphorylation by SH2 domain-containing proteins. Defective Stat signaling was observed in cells transfected with the mutant receptor found in *db/db* mice (Ghilardi et al. 1996). The role of the other isoforms is not presently known. Although Ob-Ra can mediate signal transduction *in vitro*, an *in vivo* activity has not been demonstrated (Bjorbaek et al. 1997). Ob-Ra and Ob-Rc are highly expressed in cerebral microvessels and have been suggested to be involved in leptin transport into the CNS (Banks et al. 1996; Hileman et al. 2002). Ob-Re is a soluble form of the receptor that

binds leptin in the plasma and appears to facilitate leptin action (Li et al. 1998; Huang et al. 2001).

Ob-Rb is expressed at high levels in hypothalamic neurons and has also been detected in T cells, lymph nodes, vascular endothelium, and the jejunum (Chen et al. 1996b; Ghilardi et al. 1996; Lee et al. 1996; Lord 1998; Morton et al. 1998; Sierra-Honigmann et al. 1998). Ob-Rb appears to be expressed, albeit at low levels, in most other tissues (Ghilardi et al. 1996). *In situ* hybridization detected Ob-Rb in the arcuate nucleus, dorsomedial hypothalamic nucleus (DMH), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), and the lateral hypothalamus (LH), nuclei known to be involved in regulating appetite and body weight (Hetherington and Ranson 1942; Mercer et al. 1996b; Elmquist et al. 1997; Fei et al. 1997; Elmquist et al. 1998b). Leptin injection leads to a dose-dependent activation of Stat3 specifically in the hypothalamus of wild-type and *ob/ob*, but not *db/db* mice, detectable 15 minutes after administration (Vaisse et al. 1996). Leptin also induces the hypothalamic expression of *fos*, a Stat3 target gene (Woods and Stock 1996). Leptin activates phosphatidylinositol-3-OH kinase (PI(3)K) in hypothalamic neurons, and intracerebroventricular (i.c.v.) infusion of inhibitors of this enzyme blocks leptin-induced anorexia (Niswender et al. 2001).

A number of molecules are also involved in attenuating leptin signal transduction. Leptin receptor signaling requires Jak2 phosphorylation. Leptin binding induces tyrosine phosphorylation of SHP-2, a phosphatase that decreases Jak2 phosphorylation, thereby blunting leptin signal transduction (Carpenter et al. 1998; Li and Friedman 1998). Protein-tyrosine phosphatase PTP1B also dephosphorylates Jak2 and mice lacking PTP1B are hypersensitive to leptin, have increased levels of phosphorylated Stat3, and

are resistant to obesity (Elchebly et al. 1999; Cheng et al. 2002; Cook and Unger 2002; Zabolotny et al. 2002). Ob-R signaling is also negatively regulated by SOCS-3, a member of the suppressors-of-cytokine-signaling family of molecules (Bjorbaek et al. 1998).

The high levels of Ob-Rb expression in the hypothalamus and the leptin resistance observed in rats with hypothalamic lesions indicate that leptin has direct effects on the brain (Satoh et al. 1997). I.C.V. administration of leptin reduces food intake at a dose that has no effect when delivered peripherally (Campfield et al. 1995; Stephens et al. 1995; Halaas et al. 1997). Complete depletion of adipose tissue in wild-type mice requires chronic i.c.v. infusion of only 3 ng/hr, whereas over 500 ng/hr are required when leptin is infused peripherally via a subcutaneous pump (Halaas et al. 1997). In addition, leptin has similar metabolic effects, whether delivered centrally or peripherally (Halaas et al. 1997; Kamohara et al. 1997). The leptin receptor, however, is broadly expressed and leptin has direct effects on some peripheral tissues. Leptin can modulate the immune system, angiogenesis, and bone metabolism (Lord 1998; Sierra-Honigmann et al. 1998; Ducy et al. 2000). Ob-Rb is expressed in a number of peripheral tissues and direct effects of leptin on T cells, macrophages, pancreatic β-cells, muscle, and other cell types have been reported (Shimabukuro et al. 1997a; Lord 1998; Kim et al. 2000; O'Rourke et al. 2001; Minokoshi et al. 2002). Thus, the relative importance of leptin action on brain versus peripheral sites is untested.

Neuronal circuitry regulating body weight

Leptin action in the brain modulates an increasingly complex neuronal circuit, which regulates food intake and energy expenditure (Friedman and Halaas 1998;

Schwartz et al. 2000). Ob-Rb expressing neurons in the arcuate nucleus receive inputs from other hypothalamic nuclei as well as the cortex and amygdala (DeFalco et al. 2001). In addition, hypothalamic nuclei expressing Ob-Rb have efferents that interact with the cortex, sympathetic and parasympathetic nervous systems, and pituitary.

The leptin receptor has been detected in a number of hypothalamic nuclei, such as the arcuate nucleus, VMH, DMH, and PVN, and these nuclei express neuropeptides which modulate food intake (Elmquist et al. 1997; Fei et al. 1997; Elmquist et al. 1998b). Key molecules in this neuronal circuit include neuropeptide Y (NPY) and agouti-related protein (AGRP), which stimulate food intake, and α–melanocyte stimulating hormone (α-MSH) and cocaine- and amphetamine-regulated transcript (CART), which suppress food intake. I.C.V. injection of NPY potently stimulates food intake, and NPY mRNA is upregulated in *ob/ob* mice and decreases following leptin treatment (Stanley et al. 1986; Stephens et al. 1995). *ob/ob* mice lacking NPY show an attenuation of obesity with decreased food intake and increased energy expenditure (Erickson et al. 1996). α-MSH is a cleavage product of the precursor pro-opiomelanocortin (POMC), whose cleavage also generates β- and γ-MSH, as well as adrenocorticotropin (ACTH) and β-endorphin. α-MSH acts on melanocortin receptors (MC-R), with effects on feeding through the MC-3 and MC-4 receptors. Agonists of the MC-4 receptor inhibit feeding, while antagonists stimulate feeding (Fan et al. 1997). Furthermore, MC-4 receptor knockout mice are obese and leptin resistant, as are lethal yellow, agouti (A^y) mice, a mutant strain that ectopically expresses the agouti protein in brain, which acts as an MC-4 receptor antagonist (Lu et al. 1994; Boston et al. 1997; Halaas et al. 1997; Huszar et al. 1997; Marsh et al. 1999). AGRP was cloned as an endogenous inhibitor of melanocortin signaling, and *ob/ob* mice

overexpress AGRP mRNA 8-fold (Ollmann et al. 1997; Shutter et al. 1997). In addition, transgenic mice overexpressing AGRP are obese (Graham et al. 1997). CART decreases food intake, and its expression is nearly undetectable in *ob/ob* mice, but can be induced by leptin (Kristensen et al. 1998).

Leptin signals are transduced into a neuronal response, the general nature of which is beginning to emerge. In the hypothalamus, Ob-Rb is expressed in functionally distinct classes of neurons, the best characterized being neurons that co-express either NPY or α-MSH (Mercer et al. 1996a; Cheung et al. 1997). The orexigenic molecules NPY and AGRP co-localize in arcuate nucleus neurons (Broberger et al. 1998; Hahn et al. 1998). Furthermore, POMC and CART, both of which are anorexigenic, co-localize in a distinct subset of arcuate nucleus neurons (Elias et al. 1998). NPY/AGRP neurons are inhibited by leptin, while POMC/CART neurons are activated by leptin (Stephens et al. 1995; Schwartz et al. 1997; Thornton et al. 1997; Broberger et al. 1998; Hahn et al. 1998). These neurons are thought to innervate yet to be described higher order neurons coordinating energy homeostasis.

The above model, however, is undoubtedly just one part of the higher order circuitry, as numerous additional molecules also impact feeding behavior. Melanin-concentrating hormone, galanin, orexin, peptide YY, and noradrenaline all increase food intake, while cholecystokinin (CCK), corticotropin-releasing hormone (CRH), insulin, glucagon-like peptide-1 (GLP-1), bombesin, urocortin, and serotonin all decrease food intake (Friedman and Halaas 1998).

Efferent pathways modulated by leptin

Early physiological studies demonstrated that lesions of the ventromedial hypothalamus lead to hyperphagia and obesity (Hetherington and Ranson 1942; Elmquist et al. 1999). When food intake in lesioned animals is restricted to the same levels as in controls, however, obesity still develops (Himms-Hagen 1989). Increased storage of calories in the setting of normal food intake, indicates that these animals have reduced energy expenditure. Thus, the brain serves a critical function in the coordination of energy homeostasis. The role of leptin in this process is confirmed by decreased energy expenditure in *ob/ob* mice (Boissonneault et al. 1978; Dauncey and Brown 1987). Therefore, the leptin modulated neuronal circuit, through still undescribed higher order neurons, is thought to regulate energy homeostasis.

Energy expenditure can be divided into three main components: obligate energy expenditure required for basic cellular function, physical activity, and adaptive thermogeneis (Lowell and Spiegelman 2000). Adaptive or facultative thermogenesis, is defined as heat production in response to environmental temperature or diet. Energy expenditure is generally measured by oxygen consumption, which can then be used to caculate metabolic rate. Oxygen consumption or metabolic rate increases acutely following feeding, as well as after chronic overfeeding (Shibata and Bukowiecki 1987; Sims and Danforth 1987; Levine et al. 1999). Food restriction, on the other hand is associated with a decrease in metabolic rate (Leibel et al. 1995). These alterations, though counter-productive during dieting, serve to maintain weight within a narrow range.

Leptin has clear effects on energy expenditure and is thought to exercise its effects, in part, through the autonomic nervous system. Treatment with β–adrenergic

receptor agonists causes lipolysis and an increase in energy expenditure (Galton and Bray 1967; Davies 1968; Landsberg et al. 1984). Brown adipose tissue and skeletal muscle play a major role in adaptive thermogenesis, and these tissues are highly innervated by the sympathetic nervous system. Decreased sympathetic output has been proposed to be responsible for the development of obesity (Bray and York 1998). Sympathetic outflow to brown adipose tissue is decreased in *ob/ob* mice (Young and Landsberg 1983). Leptin increases norepinephrine turnover in brown fat (Collins et al. 1996). The leptin-dependent rise in sympathetic activity to brown adipose tissue increases the expression of the uncoupling protein, UCP1 (Scarpace et al. 1997; Scarpace and Matheny 1998). Uncoupling proteins disrupt the mitochondrial proton gradient causing energy to be dissipated in the form of heat, rather than channeled into the formation of ATP. The importance of brown adipose tissue to body weight homeostasis is indicated by the development of obesity in mice with genetic ablation of brown fat (Lowell et al. 1993). UCP1 knockout mice, however, are not obese, perhaps due to compensation by other UCP isoforms (Enerback et al. 1886). Ectopic overexpression of UCP1 in white adipose tissue, on the other hand, protects mice from obesity (Kopecky et al. 1995). While UCP1 is expressed specifically in brown adipose tissue, UCP2 is expressed in most tissues, and UCP3 is expressed in muscle and brown adipose tissue (Boss et al. 1997; Fleury et al. 1997; Gimeno et al. 1997; Gong et al. 1997; Vidal-Puig et al. 1997).

Measurement of nerve activity following leptin treatment demonstrates dose-dependent increases in sympathetic output to brown adipose tissue, kidney, hindlimb, and the adrenal gland in wild-type, but not in obese Zucker rats, which have a mutation in the leptin receptor (Haynes et al. 1997). Sympathetic output acts on β-adrenergic receptors,

of which there are three well characterized types, β1, β2, and β3. Activation of these receptors leads to intracellular activation of protein kinase A (PKA) and cyclic AMP (cAMP), and these molecules are involved in energy homeostasis (McKnight et al. 1998). Disruption of RIIβ, one of the regulatory subunits of PKA, leads to an increase in free catalytic units of PKA, with greater avididity for cAMP. Despite normal food intake, these mice are lean, and show decreased triglyceride storage in white adipose tissue, elevated metabolic rate, and resistance to dietary obesity (Cummings et al. 1996). The β3-adrenergic receptor has been proposed to play a particularly important role in energy expenditure and body weight homeostasis, as its stimulation decreases adiposity and leptin expression (Collins and Surwit 1995; Mantzoros et al. 1996). The selective β3-adrenergic agonist CL316243 is effective at treating obesity and diabetes in rodents (Sum et al. 1999). In addition, a knockout of the β3-adrenergic receptor leads to a mild increase in adiposity (Susulic et al. 1995). Confirmation of the importance of β-adrenergic receptors, as opposed to an unknown target, in the regulation of body weight was difficult, however, due to functional redundancy of the different receptor types. Triple mutant mice lacking all three β-adrenergic receptors have recently been generated and demonstrate reduced metabolic rate and massive obesity on a high-fat diet, indicating that these receptors are required for diet-induced thermogenesis (Bachman et al. 2002)

Leptin also modulates energy expenditure through effects on the hypothalamic-pituitary axis. Leptin regulates many aspects of hypothalamic-pituitary function, some of which are involved in energy homeostasis (Yu et al. 1997). Thyroid hormone modulates energy expenditure, with low levels associated with decreased metabolic rate and high levels associated with increased metabolic rate (al-Adsani et al. 1997). Leptin

administration can prevent fasting-induced suppression in thyroid hormone levels, by blunting the suppression in prothyrotropin releasing hormone RNA levels in the hypothalamic PVN (Ahima et al. 1996; Legradi et al. 1997). Elevated glucocorticoid levels, as in Cushing's syndrome, are associated with increased fat deposition. In fact, transgenic mice overexpressing 11β hydroxysteroid dehydrogenase type 1 (11β HSD-1) have increased adipose tissue levels of corticosterone and develop visceral obesity and features of the metabolic syndrome (Masuzaki et al. 2001). Glucocorticoid levels are elevated in *ob/ob* and *db/db* mice, and obesity in these animals is improved by adrenalectomy or treatment with glucocorticoid antagonists (Naeser 1973; Freedman et al. 1986). Elevated glucocorticoid levels are unique to *ob/ob* and *db/db* mice, whereas other forms of rodent obesity are not associated with elevated corticosterone (Bray and York 1979). As further evidence for the role of leptin in modulating corticosterone levels, fatless A-ZIP/F-1 lipoatrophic mice, which are deficient in circulating leptin, also have hypercorticosteronemia (Haluzik et al. 2002). Leptin has been shown to lower glucocorticoids both by direct action on the adrenals and via the hypothalamic PVN (Bornstein et al. 1997; Huang et al. 1998; Cherradi et al. 2001).

The growth hormone (GH) axis is also perturbed in obesity (Scacchi et al. 1999). In humans, plasma GH is inversely related to measures of adiposity, and in humans and rodents, obesity is also associated with reduced plasma insulin-like growth factor I (IGF-I), which is downstream of GH (Rudman et al. 1981) (Copeland et al. 1990; Zhou et al. 1998a). In obese Zucker rats, GH levels and pulsatility are severely decreased, due to decreased hypothalamic growth-hormone releasing factor (GRF) and an impaired pituitary response to GRF (Finkelstein et al. 1986; Tannenbaum et al. 1990). Transgenic

rats expressing a low level of human GH show suppression of endogenous GH secretion and pulsatility and develop massive obesity and insulin resistance (Ikeda et al. 1997). Double-labeling immunohistochemistry detected ObR in some hypothalamic neurons containing GH-releasing hormone (GHRH) (Hakansson et al. 1998). Administration of a leptin antiserum to rats leads to decreased GH secretion, suggesting that leptin, may, in part, regulate GH secretion (Carro et al. 1997). Furthermore, central leptin infusion stimulates spontaneous pulsatile GH secretion, as well as secretion in response to GHRH (Tannenbaum et al. 1998).

Metabolic effects of leptin

Differential actions of leptin and food restriction

Leptin regulates energy expenditure by exerting specific and unique metabolic effects. These effects are likely due to modulation of sympathetic outflow and neuroendocrine activity, as well as direct actions of leptin on peripheral tissues. Rodents deficient in leptin (*ob/ob*) or resistant to its action (*db/db, fa/fa*) are markedly hyperphagic and morbidly obese. However, the effects of leptin deficiency are not purely the result of increased caloric intake, as food restricted *ob/ob* mice still develop obesity (Coleman 1978). Replacement of leptin in *ob/ob* mice leads to the specific depletion of fat mass, whereas food restriction depletes both fat and lean mass (Halaas et al. 1995; Pelleymounter et al. 1995; Halaas et al. 1997). *ob/ob* mice that have been food restricted to the level that leptin treated mice voluntarily consume (pair-fed) show smaller decreases in body weight and size of adipose depots (Levin et al. 1996). In addition, food

restriction is associated with a compensatory decrease in energy expenditure, which does not occur in response to leptin-induced hypophagia (Halaas et al. 1997). Finally, whereas food restriction leads to a rise in serum free fatty acids, leptin-mediated weight loss is not associated with a rise in free fatty acids or ketones, suggesting a unique mechanism of fatty acid oxidation (Chen et al. 1996a; Shimabukuro et al. 1997a). Taken together, these data confirm that leptin's effects on body weight are not due to its anorectic effects alone, but must also require actions on peripheral metabolism.

Effects on lipid metabolism

In the absence of effective leptin action, triglyceride not only accumulates in adipose tissue, but also in other peripheral sites such as liver, skeletal muscle, heart, and pancreatic islets. One dramatic manifestation can be seen in *ob/ob* liver, which is massively enlarged and steatotic, with numerous lipid-filled vacuoles apparent on histological examination (Figure 1.5). This build-up of lipid in non-adipose tissues causes lipotoxicity, which contributes to insulin resistance and organ dysfunction in the obese state (Unger 2002). Increased intracellular lipid in insulin-responsive tissues is increasingly appreciated as being critical to the development of insulin-resistance and diabetes (McGarry 1992; Shulman 2000). In addition, lipid accumulation in islets is involved in islet hypoplasia and destruction in advanced diabetes, via increased synthesis of the apoptotic mediators nitric oxide and ceramide, and reduced levels of Bcl-2, an inhibitor of apoptosis (Lee et al. 1994; Shimabukuro et al. 1997b; Shimabukuro et al. 1998a; Shimabukuro et al. 1998b; Shimabukuro et al. 1998c).

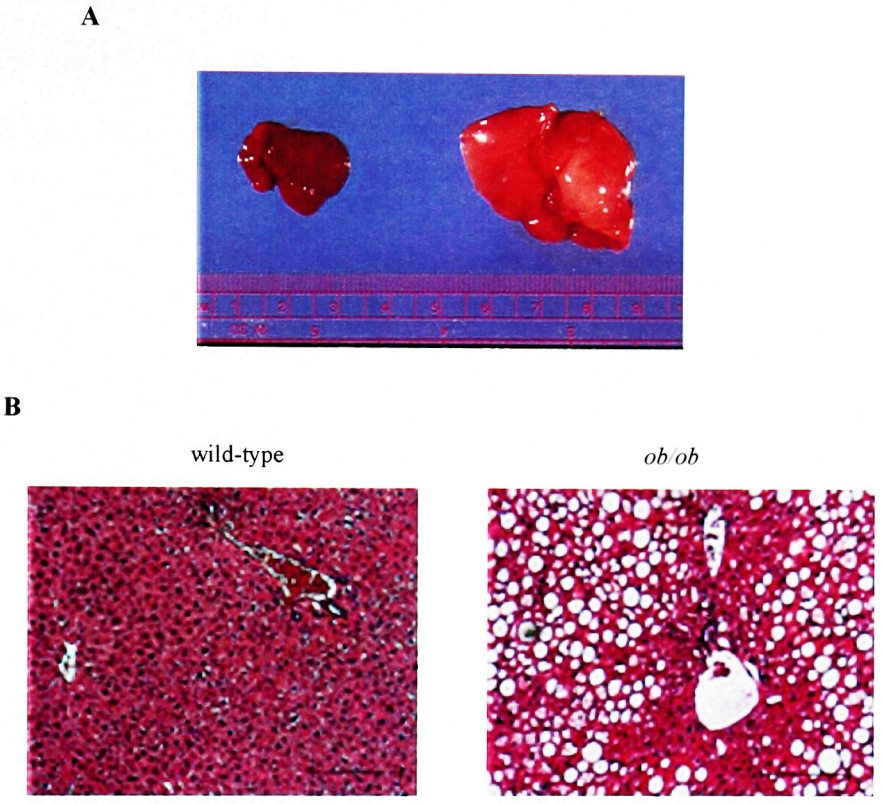


Figure 1.5 Hepatomegaly and fatty liver in *ob/ob* mice. (A) Photographs of freshly dissected livers, wild-type on left and *ob/ob* on right. The *ob/ob* liver is 3-4x larger and has a shiny appearance, consistent with lipid accumulation. (B) Hematoxylin and eosin stained liver sections demonstrate vacuolation throughout *ob/ob* liver, suggestive of lipid accumulation and fatty liver disease.

Given the deleterious consequences of lipid accumulation in peripheral tissues, evidence suggests a role for leptin in the control of lipid homeostasis in nonadipose tissues (Unger 2002). When caloric intake exceeds energy expenditure, leptin levels rise to prevent deposition of lipid in peripheral sites. In states of leptin deficiency or leptin resistance, however, this action is defective and lipotoxic disease develops. In support of this hypothesis, leptin treatment, significantly more so than pair-feeding, rapidly reduces lipid in non-adipose sites by decreasing triglyceride formation and increasing free fatty acid oxidation (Shimabukuro et al. 1997a). Islets isolated from hyperleptinemic rats, as compared to islets isolated from pair-fed or untreated rats, show a markedly increased ratio of fatty acid oxidation to esterification (Zhou et al. 1997). Relative to control and pair-fed samples, these islets demonstrate increased expression of acyl CoA oxidase (ACO) and carnitine palmitoyltransferase-I (CPT-I), enzymes involved in fatty acid oxidation, as well as increased expression of UCP-2. In addition, islets from both leptin treated and pair-fed rats show a comparable reduction in the expression of acetyl-CoA carboxylase (ACC) and glycerol-3-phospate acyltransferase (GPAT), enzymes involved in fatty acid biosynthesis and esterification (Zhou et al. 1997). Adipose tissue demonstrates a similar pattern of gene expression in hyperleptinemic animals, along with loss of expression of the adipocyte markers fatty acid binding protein-2 (aP2), tumor necrosis factor α, and leptin, and appearance of the preadipocyte marker Pref-1 (Zhou et al. 1999).

The biochemical pathways mediating leptin's effects on fatty acid oxidation are beginning to be elucidated. The nuclear hormone receptor PPARα, which regulates many

of the enzymes of fatty acid oxidation, has been proposed to coordinate this transcriptional program (Aoyama et al. 1998). Hyperleptinemia in wild-type, but not *fa/fa* rats, induces the expression of PPARα, ACO, and CPT-I in peripheral tissues, genes which likely mediate the effects of leptin (Zhou et al. 1998b; Zhou et al. 1999). Administration of leptin to PPARα knockout mice leads to a smaller reduction in adipose depots and a smaller depletion in liver triglyceride, confirming that this pathway is required for the lipopenic effects of leptin (Lee et al. 2002). In muscle, leptin has been found to stimulate fatty acid oxidation, both directly and secondary to signaling in the brain. These actions are mediated through activation of α2 AMP-kinase, which phosphorylates and inhibits acetyl-CoA carboxylase (ACC) (Minokoshi et al. 2002). Inhibition of ACC leads to reduced levels of malonyl CoA, which is required for fatty acid biosynthesis and also inhibits the mitochondrial carnityl palmityl transferase system, the rate-limiting step in the import and oxidation of fatty acids in mitochondria (McGarry et al. 1977). Thus, malonyl CoA is considered to be a crucial metabolic control point, with reduced levels leading to decreased fatty acid biosynthesis and increased fatty acid oxidation (Ruderman et al. 1999). This mechanism also accounts for the lean phenotype and increased fatty acid oxidation in mice lacking acetyl-CoA carboxylase 2 (Abu-Elheiga et al. 2001). However, activation of α2 AMP-kinase is not associated with increased leptin-mediated fatty acid oxidation in all tissues. While leptin stimulates fatty acid oxidation in liver and heart, α2 AMP-kinase is not activated, suggesting that alternative mechanisms can also explain leptin's lipopenic actions (Atkinson et al. 2002; Lee et al. 2002).

Leptin's effects on lipid metabolism are not solely the consequence of enhanced fatty acid oxidation, but are also due to decreased lipogenesis. *ob/ob* and *db/db* mice have enlarged steatotic livers (Yen et al. 1976; Koteish and Diehl 2001). The fatty liver is, in part, the result of an increased rate of hepatic lipogenesis, which is also thought to contribute to the development of obesity (Kaplan and Leveille 1981). Leptin treatment normalizes the hepatomegaly and associated elevations in hepatic lipid (Levin et al. 1996). Analysis of *ob/ob* livers by ^{13}C NMR after five days of leptin treatment reveals decreased *de novo* fatty acid synthesis, which is supported by biochemical assays showing decreased ACC and fatty acid synthase (FAS) activity (Cohen et al. 1998). Thus, by modulating gene expression and the pathways of fatty acid biosynthesis and oxidation, leptin confines storage of triglycerides to adipose tissue and prevents lipotoxic disease during states of overnutrition.

More recently, leptin's metabolic effects have been shown to be important in lipodystrophies, a rare set of diseases characterized by complete or near-complete loss of adipose tissue (Garg 2000; Reitman et al. 2000). In the absence of adipose tissue, ingested nutrients are deposited as triglycerides in liver, muscle, and other peripheral tissues leading to severe insulin resistance and diabetes. Transgenic overexpression in white adipose tissue of either nuclear sterol regulatory element-binding protein-1c (*aP2-SREBP-1c tg*) or a dominant negative protein preventing the binding of certain bZIP transcription factors (A-ZIP) produces mice with a near complete absence of adipose tissue, along with marked hepatic steatosis, insulin resistance, and hyperglycemia (Moitra et al. 1998; Shimomura et al. 1998a). In the near-absence of adipose tissue, circulating levels of leptin and other adipose-derived factors are extremely low, and a deficiency in

one or more of these factors has been hypothesized to be responsible for disease. Treatment of *aP2-SREBP-1c tg* lipodystrophic mice with physiological doses of leptin for 12 days led to a complete normalization of hepatic steatosis and glucose and insulin levels, while pair-feeding had much more modest effects (Shimomura et al. 1999c). Originally, treatment of A-ZIP mice (which have a more severe phenotype) with leptin produced more modest effects on the diabetic phenotype (Gavrilova et al. 2000b). However, later studies showed that supraphysiological levels of leptin can correct the phenotype in these mice (Ebihara et al. 2001). In addition, surgical implantation of fat from wild-type mice, but not from *ob/ob* mice, can fully rescue the phenotype of A-ZIP mice (Gavrilova et al. 2000a; Colombo et al. 2002). While these findings do not rule out a role for other adipose factors, leptin clearly plays a crucial role in depleting peripheral triglyceride and correcting diabetes in these mice. Based on these findings, a leptin trial was initiated in lipodystrophic humans, resulting in marked reductions in appetite, hepatic steatosis, muscle triglyceride, diabetes, and hypertriglyceridemia (Oral et al. 2002; Petersen et al. 2002). As lipodystrophy is a side effect of antiretroviral therapy for HIV, leptin therapy may become useful for this growing population of patients (Estrada et al. 2002).

Effects on carbohydrate metabolism

Leptin also has well-documented effects on glucose metabolism. *ob/ob* and *db/db* mice are hyperglycemic and hyperinsulinemic, with marked insulin resistance. The hyperglycemia and hyperinsulinemia of *ob/ob* mice are corrected with leptin administration, and leptin treatment leads to a greater reduction in glucose, insulin, and

hepatic glycogen than pair-feeding (Pelleymounter et al. 1995; Levin et al. 1996). Obesity is associated with hyperinsulinemia, which is the body's attempt to compensate for worsening insulin resistance. After a certain threshold, the capacity of the pancreatic islets is exceeded, at which point, hyperinsulinemia fails to compensate for insulin resistance, and hyperglycemia and diabetes become fully evident. Thus, the modulation of glucose metabolism by leptin is influenced by a complex interplay between obesity, leptin, and insulin. Leptin receptors are expressed in pancreatic β cells and treatment of islets inhibits insulin secretion by activating ATP-sensitive potassium channels (Kieffer et al. 1996; Harvey et al. 1997; Kieffer et al. 1997). As well as inhibiting insulin secretion, leptin counters hyperinsulinemia by decreasing the expression of preproinsulin mRNA in *ob/ob* islets (Seufert et al. 1999a). Leptin has similar effects on insulin secretion and gene expression in islets from human donors (Seufert et al. 1999b). As insulin, in turn, increases the expression of leptin mRNA, the existence of an adipoinsular axis has been proposed, where insulin stimulates leptin production, and leptin, in turn, inhibits insulin production (Saladin et al. 1995). Therefore, leptin's effects on glucose metabolism may, in large part, be mediated via its interactions with insulin.

A number of studies, however, point to a more specific role for leptin in carbohydrate metabolism. When streptozotocin-induced, insulin-deficient diabetic rats were treated with leptin for 12-14 days and studied with a hyperinsulinemic-euglycemic clamp, leptin, independent of its effects on food intake, restored euglycemia and peripheral insulin sensitivity, and improved post-absorptive glucose metabolism (Chinookoswong et al. 1999). Transgenic overexpression of high levels of leptin leads to a complete disappearance of white fat and increased glucose metabolism and insulin

sensitivity (Ogawa et al. 1999). These mice are strikingly different from lipoatrophic mice, which also have no fat, but instead develop steatosis and severe diabetes (Moitra et al. 1998; Shimomura et al. 1998a).

Acute treatment with leptin, either as a five hour intravenous or i.c.v infusion, or even as a single microinjection into the ventromedial hypothalamus, similarly increased glucose uptake and turnover in skeletal muscle and brown adipose tissue. Leptin stimulated glucose uptake was diminished in denervated muscle, pointing to centrally orchestrated sympathetic output as a mediator of these effects (Kamohara et al. 1997; Minokoshi et al. 1999). The increased glucose turnover and uptake occurred in the setting of decreasing hepatic glycogen and constant plasma insulin and glucose, suggesting that leptin acutely increases hepatic gluconeogenesis from glycogen to compensate for enhanced glucose turnover (Kamohara et al. 1997). An acute intravenous infusion of leptin in *ob/ob* mice also increased glucose uptake and turnover in brown adipose, heart, and brain, but not in white adipose or skeletal muscle. This protocol did not influence hepatic lipid metabolism, but did stimulate hepatic glucose production, and was associated with increased activity of glucose-6-phosphatase (G6Pase) and decreased activity of phosphoenolpuruvate carboxykinase (PEPCK) (Burcelin et al. 1999).

An acute leptin infusion in lean rats clamped at physiological hyperinsulinemia, however, found no difference in glucose uptake and glycogen synthesis between leptin treated and control animals. In this study, leptin enhanced insulin suppression of hepatic glucose production and redistributed intrahepatic glucose fluxes such that the contribution from glycogenolysis was reduced and that from gluconeogenesis was enhanced, changes marked by decreased levels of glucokinase RNA and increased levels

of PEPCK RNA (Rossetti et al. 1997). This group reported the same findings with i.c.v. leptin, again indicating that leptin's effects on glucose metabolism are centrally mediated (Liu et al. 1998). A subsequent insulin clamp study compared the effects of 8 days of leptin and pair-feeding on glucose metabolism and showed that leptin enhanced insulin-mediated inhibition of hepatic glucose production with a similar repartitioning of glucose fluxes, while also increasing insulin stimulated peripheral glucose uptake. Relative to food restriction, chronic administration of leptin also decreased expression of glucokinase and increased expression of G6Pase and PEPCK (Barzilai et al. 1997). While the insulin clamp experiments suggest that leptin mainly augments insulin action, the shift in glucose fluxes and the changes in gene expression in a direction counter to that induced by insulin alone, indicate that leptin has novel effects on glucose metabolism, independent of insulin. ^{13}C NMR studies demonstrated that five days of leptin treatment in *ob/ob* mice increases hepatic gluconeogenesis and also increases the rate of hepatic glycogen synthesis, in part by increasing the activity of glycogen synthase (Cohen et al. 1998). Discrepancies exist in these studies, which may be due to the use of different assay techniques and rodent species, or due to different effects of leptin in lean and insulin-resistant obese animals or following acute versus chronic administration

Other mediators of energy homeostasis

While leptin is critical in the regulation of energy homeostasis, a number of other circulating factors also act in endocrine pathways involved in energy metabolism. Insulin, which is secreted by pancreatic β cells, acts peripherally, as an anabolic hormone, stimulating glucose uptake and lipogenesis. However, insulin also crosses the blood-brain

barrier, and insulin receptors are expressed in brain regions involved in feeding (Baskin et al. 1988; Baura et al. 1993). High insulin levels reduce food intake and body weight, while low levels stimulate food intake (Woods et al. 1979; Sipols et al. 1995). Mice with a neuronal-specific knockout of the insulin receptor are hyperphagic and develop diet-induced obesity (Bruning et al. 2000). Similarly, rats with decreased levels of hypothalamic insulin receptors are hyperphagic and insulin resistant (Obici et al. 2002).

The recently identified hormone ghrelin, is synthesized by the stomach and the hypothalamus, and acts as a ligand for the growth hormone secretagogue receptor (GHS-R) (Kojima et al. 1999). Ghrelin not only induces growth hormone secretion, but also stimulates food intake, with peripheral or central administration causing weight gain due to hyperphagia and reduced utilization of fat (Tschop et al. 2000; Wren et al. 2000; Nakazato et al. 2001; Wren et al. 2001). Interestingly, plasma ghrelin levels rise after dieting, but remain low following gastric bypass surgery, explaining the vastly different success rates of these two weight loss treatments (Cummings et al. 2002).

A number of adipose-derived factors, in addition to leptin, have peripheral metabolic effects, challenging the traditional view of adipose tissue as a passive repository of triglyceride (Trayhurn and Beattie 2001). Levels of TNFα, a factor mediating cachexia, are increased in different rodent models of obesity, and neutralization of TNFα increases peripheral glucose uptake in response to insulin (Oliff et al. 1987; Hotamisligil et al. 1993). TNFα causes insulin resistance by inducing serine phosphorylation of insulin receptor substrate 1 (IRS-1), converting IRS-1 into an inhibitor of insulin receptor tyrosine kinase activity (Hotamisligil et al. 1996). This inhibitory form of IRS-1, which can attenuate signaling through the insulin receptor, was

observed in muscle and adipose tissue from obese *fa/fa* rats (Hotamisligil et al. 1996). Deletion of the TNFα gene or the two genes encoding its receptor improved insulin sensitivity in lean mice, diet-induced obese mice, gold-thioglucose injected mice, and *ob/ob* mice, further supporting the pivotal role of TNFα in obesity-associated insulin resistance (Uysal et al. 1997; Ventre et al. 1997). Others, however, have found that rather than contributing to insulin resistance, TNF receptors protect against obesity-related diabetes (Schreyer et al. 1998).

Other factors include resistin, which is secreted from adipose tissue and circulates at increased levels in models of dietary and genetic obesity (Kim et al. 2001; Steppan et al. 2001). Treatment of mice with resistin leads to impaired glucose tolerance and insulin action, while administration of anti-resistin antibodies improves hyperglycemia and insulin resistance in diet-induced obese mice (Steppan et al. 2001). The specific physiological role of resistin, however, awaits the identification of its site of action and the generation of a knockout mouse. Acrp30 (adipocyte complement related protein of 30kDa or adiponectin) is an abundant serum protein that can form large oligomers (Scherer et al. 1995). Treatment of mice with a proteolytic cleavage product of Acrp30 induces fatty acid oxidation and weight loss (Fruebis et al. 2001). In addition, Acrp30 inhibits hepatic glucose production, augments hepatic insulin action, and reverses insulin resistance due to lipoatrophy and obesity (Berg et al. 2001; Combs et al. 2001; Yamauchi et al. 2001). Obese type 2 diabetics have low adiponectin levels and adiponectin mutations have been associated with diabetes (Weyer et al. 2001; Kondo et al. 2002). Two separate groups have generated adiponectin null mice, and in one study, but not in the other, adiponectin deficiency leads to diet-induced insulin resistance (Ma et al. 2002;

Maeda et al. 2002). Thus, the precise function of Acrp30 remains to be elucidated. Other adipose-derived factors including angiotensinogen, adipsin, acylation-stimulating protein, haptoglobin, retinol-binding protein, interleukin 6, plasminogen activator inhibitor-1 and tissue factor also play important, but less well-defined roles in inflammation, metabolism, and energy homeostasis (Trayhurn and Beattie 2001).

In addition to adipose tissue, other peripheral tissues such as liver, muscle, and pancreas may also secrete factors that modulate peripheral metabolism. Myostatin is secreted from skeletal muscle and negatively regulates muscle mass, as mice and cattle lacking myostatin display muscle hypertrophy and hyperplasia (Grobet et al. 1997; McPherron et al. 1997; McPherron and Lee 1997). In addition, myostatin null mice have reduced adiposity, in the setting of normal food intake, and double mutant *ob/ob* or A^y mice also lacking myostatin show a partial correction of obesity and diabetes (McPherron and Lee 2002). Systemic overexpression of myostatin, on the other hand, induces muscle and fat wasting, resembling cachexia (Zimmers et al. 2002).

Leptin and body weight homeostasis in humans

Human obesity mutations

The scarcity of identified human mutations in the leptin circuit supports the premise that obesity is the result of a complex constellation of genetic and environmental factors, many of which have yet to be elucidated. While a number of genetic studies have identified linkages to loci contributing to obesity in humans, monogenic forms of obesity are rare (Barsh et al. 2000). However, study of these mutations illustrates a remarkable similarity to the homologous mouse mutants. Mutations in the human *ob* gene are thought

to be rare, as numerous studies in obese subjects have failed to detect any mutations (Maffei et al. 1996; Barsh et al. 2000). Thus far, two kindreds with homozygous loss-of-function leptin mutations have been identified, displaying hyperphagia, morbid obesity, and hypogonadotropic hypogonadism (Montague et al. 1997; Strobel et al. 1998). One family has been identified with a mutation producing a truncated leptin receptor, and these patients have a similar phenotype to individuals lacking leptin, with the additional presence of growth retardation and central hypothyroidism (Clement et al. 1998). Unlike rodents with mutations in leptin or its receptor, cold intolerance, severe diabetes, and increased glucocorticoid production have not been reported in human mutants. Two children with a phenotype of obesity, adrenal insufficiency, and hypopigmentation have also been identified and shown to have loss-of-function POMC mutations (Krude et al. 1998). Obese humans with dominant mutations in the MC-4 receptor have also been described, and this is the most commonly mutated gene in human obesity, with 3-5% of humans with BMI > 40 estimated to have mutations in this gene (Vaisse et al. 1998; Yeo et al. 1998; Hinney et al. 1999).

The identification of humans with loss-of-function mutations in leptin or POMC suggests that hormone replacement therapy might be an effective treatment. One of the leptin-deficient probands has been treated with leptin for over a year with daily subcutaneous injections, with a notable decrease in body weight and appetite, along with pubertal progression (Farooqi et al. 1999). The successful use of leptin therapy in these children indicates that leptin is capable of enacting the same physiological response in humans as in mice.

Leptin and the pathogenesis of obesity

Although specific mutations in the leptin loop are rare in humans, both obesity and other eating disorders are associated with perturbations in circulating leptin. Modulation of circulating leptin levels, in a manner that would have been adaptive in prehistoric times where nutrients were scarce, is now maladaptive and contributes to the high failure rate of dieting. Weight loss in overweight individuals is associated with a decrease in leptin levels, which consequently stimulates appetite and reduces energy expenditure, tending to restore body weight to its original value (Leibel et al. 1995; Maffei et al. 1995b; Considine et al. 1996). Low dose leptin administration during dieting can prevent these effects associated with falling leptin, and may help dieters maintain reduced body weight (Rosenbaum et al. 2002). Alterations in circulating leptin levels are also involved in the pathogenesis of anorexia nervosa. These patients have very low leptin levels, and the rapid rise in leptin following refeeding may play a role in this disorder by diminishing appetite before normal weight is reached (Casanueva et al. 1997; Mantzoros et al. 1997).

Human obesity, though largely polygenic, is stll characterized by altered leptin physiology and can be broadly categorized as resulting from either inappropriately low leptin levels or leptin resistance. These two possibilities can be distinguished by measurement of plasma leptin. With the exception of *ob/ob* mice and rare human cases, leptin levels are high in most rodent and human obesity, illustrating the prevalence of leptin resistance in the pathogenesis of obesity (Maffei et al. 1995a; Maffei et al. 1995b; Considine et al. 1996). Rodent models have facilitated examination of leptin resistance on a molecular level. Peripheral and central administration of leptin to A^y, New Zealand

obese (NZO), and diet-induced obese (DIO) AKR/J mice confirmed their resistance to leptin (Halaas et al. 1997; Van Heek et al. 1997). A^y mice are totally resistant to high peripheral and i.c.v. doses of leptin, suggesting that signaling through the MC-4 receptor is necessary for the response to leptin and for maintenance of normal body weight (Halaas et al. 1997; Marsh et al. 1999). NZO mice are resistant to peripheral leptin, but respond normally to centrally delivered leptin, indicating that this polygenic form of obesity may be the result of impaired leptin transport into the CNS (Halaas et al. 1997). In obese, hyperleptinemic humans, a decreased cerebrospinal fluid to serum leptin ratio has been noted, supporting the existence of a saturable system for leptin transport into the brain, defects in which might account for leptin resistance (Caro et al. 1996; Schwartz et al. 1996). Although DIO mice respond to central leptin, they show peripheral resistance, requiring high doses to effect weight loss (Halaas et al. 1997; Van Heek et al. 1997). DIO mice are normally lean, but become obese when fed a high-fat diet, while other strains can maintain a stable weight on such a diet (West et al. 1992). Determining the mechanism whereby a high fat diet, on certain genetic backgrounds, contributes to obesity, would be highly relevant to human obesity, judging by the increased prevalence of obesity in populations consuming high fat, western diets. One proposed mechanism for diet-induced obesity holds that a palatable, high-fat diet causes a temporary weight gain, which is then normally lost. In susceptible individuals, however, the increased leptin levels associated with weight gain could desensitize the leptin response and bring about a new 'set-point' at a higher weight (Friedman and Halaas 1998).

Decreased leptin synthesis or secretion could also lead to obesity. Although most obese humans are hyperleptinemic, 5-10% have relatively low leptin levels, pointing to

decreased leptin as being relevant in a subset of obesity (Maffei et al. 1995b; Considine et al. 1996). Relatively low leptin levels have been detected in Pima Indians, prior to the development of obesity (Ravussin et al. 1997). *ob/ob* mice carrying a human leptin transgene, expressing constitutively low levels of leptin, develop a marked obesity, which is not as severe as that in *ob/ob* mice, and which can be similarly corrected by leptin administration (Ioffe et al. 1998). In addition, humans with partial leptin deficiency also tend to develop increased adiposity (Farooqi et al. 2001).

Molecular targets for the regulation of body weight

Current and future obesity therapy

Given the immense health burden of obesity, finding new, effective treatments is imperative. While the public would welcome a therapy that could eradicate obesity, studies indicate that even a 10% weight loss can decrease complications from obesity such as diabetes, hypertension, and hyperlipidemia, and increase longevity (Goldstein 1992). Currently, the most widely used interventions are diet and exercise, weight loss drugs, and bariatric surgery (Yanovski and Yanovski 2002).

Considering the overwhelming prevalence of weight regain following dieting and the risks of surgery, pharmacotherapy for obesity seems to be a reasonable approach. Both leptin and drugs targeting components of the leptin circuit are currently in clinical trials (Crowley et al. 2002). While leptin treatment of obese humans leads to a dose-dependent weight loss, its effects are rather modest, which is to be expected since most obese humans are leptin resistant (Heymsfield et al. 1999). However, leptin appears to be far more effective in treating leptin-deficient obese and lipodystrophic patients, and may

also be useful in obese individuals with inappropriately low levels of leptin (Farooqi et al. 1999; Farooqi et al. 2001; Oral et al. 2002). The development of novel therapies for obesity requires a fuller understanding of the physiological basis of body weight regulation. As leptin is a central mediator of energy homeostasis, further elucidation of its effects on appetite and metabolism will shed light on the biology of obesity.

Specific aims

This thesis aims to further define the molecular basis for leptin's metabolic effects. The effects of leptin on the liver are the focus here, due to this organ's critical role in coordinating peripheral metabolism. The physiological role of central versus peripheral sites of leptin action was examined using tissue-specific knockout mice (Chapter 3). These studies indicated that central action of leptin is critical for its effects on body weight and hepatic lipid metabolism. Next, transcriptional profiling was used to identify molecular targets of leptin's specific effects on peripheral metabolism (Chapter 4). Using a novel computational approach, genes whose expression was specifically modulated during leptin-mediated weight loss were identified. One of these genes, stearoyl-CoA desaturase-1 (SCD-1) was shown to be required for leptin-mediated weight loss (Chapter 5). *ob/ob* mice lacking SCD-1 were resistant to obesity and showed a correction of the hypometabolic phenotype and hepatic steatosis characteristic of leptin-deficiency. Mice lacking SCD-1 were also found to be resistant to alcoholic and nonalcoholic fatty liver disease (Chapter 6). Other leptin-regulated genes were also identified and studies on the role of insulin-like growth factor binding protein-2 (IGFBP-

2) in leptin-mediated weight loss are presented (Chapter 7). Finally, a summary of the findings and directions for future research are discussed (Chapter 8).

Materials and Methods from Chapter 3

ObR floxed mice. A 12 kb genomic clone containing the first coding exon of ObR was isolated. A single loxP element was PCR amplified from the plasmid pNeoTKLox. The oligonucleotide primers were engineered to introduce a BamHI-NcoI double restriction site at one end and a BamHI site at the other end. Following digestion with BamHI, the product was ligated into the BamHI site of the genomic clone, resulting in the clone BNLOXB with a single LoxP site upstream of the first exon. A loxP-Neo-HSV-TK-LoxP element was isolated by digesting pNeoTKLox with BstXI and SalI. The fragment was blunt-ended and cloned into Bgl-II digested blunt-ended BNLOXB. This clone, denoted ObR-lox-SS1-lox, was double digested with AflII and KpnI, blunt-ended, and religated to generate the targeting vector. The excised 500 bp AflII-KpnI fragment was used as Probe 1. The targeting vector was linearized with SalI and electroporated into 129/SV ES cells. Cells were selected with G418 and surviving clones were screened for homologous recombination. Positive clones were transiently transfected with the Cre recombinase expressing plasmid, p0G231 (kindly provided by S. O'Gorman) (O'Gorman et al. 1997). Cells were then subjected to gancyclovir selection and surviving clones were checked by Southern blotting using Probe 2, which is within exon 2, to confirm the deletion of the Neo-HSV-TK cassette (Type II deletion). ES cells with the correct genotype ($ObR^{flox/+}$) were injected into C57Bl/6 blastocysts, and resulting chimeras were bred with C57Bl/6 mice to obtain germline transmission. $ObR^{flox/+}$ mice were crossed to generate the line of ObR floxed mice, denoted $ObR^{flox/flox}$.

ObR null mice. $ObR^{flox/+}$ mice were crossed with transgenic adenovirus EIIA cre mice (kindly provided by H. Westphal) (Lasko et al. 1996). Since Cre is expressed early in embryogenesis in this line, progeny were screened by Southern blotting with Probe 2 for germline Cre-mediated deletion of the first coding exon (Type I deletion). These mice, with the genotype $ObR^{\Delta/+}$, were crossed to produce homozygous $ObR^{\Delta/\Delta}$ mice, designated ObR null mice.

Conditional deletion of ObR. Neuron-specific deletion of ObR was achieved using synapsinI-Cre transgenic mice (SynI-Cre(+)) (kindly provided by J. Marth) (DeFalco et al. 2001; Zhu et al. 2001). Hepatocyte-specific deletion was achieved using albumin-cre transgenic mice (Alb-Cre(+)). The Alb-Cre transgene (Figure 3.5A) was constructed using plasmid NB, which contains 2 kb of the albumin promoter-enhancer (kindly provided by R. Palmiter) (Pinkert et al. 1987). A 2 kb fragment containing the Cre-recombinase gene and a nuclear localization and polyadenylation signal was excised from a separate plasmid by digesting with BglII and blunt-ending and then digesting with KpnI. This fragment was cloned into KpnI-EcoRV digested plasmid NB downstream of the albumin promoter-enhancer. The resulting plasmid was digested with NotI and KpnI to release the 4 kb transgene, which was gel purified and injected into fertilized eggs from C57/Bl6 x CBA (F1) mice to produce transgenic mice. The presence of the transgene in both (SynI-Cre(+)) and (Alb-Cre(+)) mice was verified by PCR and Southern blotting. Tissue-specific knockout mice were generated by two successive crosses (Figure 3.2a). First, $ObR^{\Delta/+}$ mice were crossed with either synapsinI-Cre transgenic mice (SynI-Cre(+)) or albumin-cre transgenic mice (Alb-Cre(+)) to generate $ObR^{\Delta/+}$, SynI-Cre(+) mice or $ObR^{\Delta/+}$, Alb-Cre(+) mice. These mice were then mated to

$ObR^{flox/flox}$ mice to generate mice with the genotype $ObR^{\Delta/flox}$, SynI-Cre (+) or $ObR^{\Delta/flox}$, Alb-Cre (+), hereafter designated $ObR^{SynI}KO$ and $ObR^{Alb}KO$ respectively. Mice with the following genotypes were also generated: (1) $ObR^{\Delta/flox}$, SynI-Cre(-) and $ObR^{\Delta/flox}$, Alb-Cre(-) (heterozygotes), (2) $ObR^{flox/+}$, SynI-Cre (+) and $ObR^{flox/+}$, Alb-Cre (+) (referred to as wild type), and (3) $ObR^{flox/+}$, SynI-Cre (-) and $ObR^{flox/+}$, Alb-Cre (-) (referred to as wild type). All animals here, and those described in other chapters, were housed under controlled temperature (23° C) and lighting (12 hours of light, 0700-1900 hours; 12 hours of dark, 1900-0700) with free access to food and water, unless otherwise indicated. All procedures were in accordance with the guidelines of the Rockefeller University Laboratory Animal Research Center.

Assay of Cre specificity. Genomic DNA was prepared from multiple tissues from $ObR^{flox/+}$ SynI-Cre (+) and $ObR^{flox/+}$ Alb-Cre (+) mice and 200 ng was PCR amplified using primers flanking the first coding exon of ObR. A schematic of the primer locations is shown in (Figure 3.2b). In tissues expressing Cre recombinase, exon 1 is excised and a single loxP site remains, generating an ObR^{Δ} allele. While primers 1 and 3 can amplify a product from the ObR^{Δ} allele, the primers are separated by too great a distance for amplification to occur in the ObR^{flox} or ObR^{+} (wild-type) alleles. As a control, primers 1 and 2 were used to amplify both the ObR^{flox} and ObR^{+} alleles from all tissues. The ObR^{+} allele produces a slightly smaller product due to the absence of loxP sequence. The primer sequences are as follows: primer 1- 5' GTCACCTAGGTTAATGTATTC 3', primer 2- 5' TCTAGCCCTCCAGCACTGGAC 3', primer 3- 5' GCAATTCATATC AAAACGCC 3'.

Assay of Cre efficiency. To determine the degree of Cre-mediated recombination, ObR expression levels from all $ObR^{SynI}KO$ and $ObR^{Alb}KO$ mice and a sample of mice with all other possible genotypes were quantitated using Taqman® real time PCR. Total RNA was isolated from $ObR^{SynI}KO$ and $ObR^{Alb}KO$ mice and reverse transcribed into cDNA using random hexamers with Reverse Transcription Reagents from Roche (Branchburg, NJ). Expression levels were determined using 25 ng of each cDNA sample assayed in duplicate and amplified with the ABI Prism 7700® Sequence Detection System (Perkin Elmer Applied Biosystems, Foster City, CA). The location of primers and fluorescent probes is indicated in Figure 3.3b. As amplification occurs, the probe is cleaved, resulting in a signal from a reporter dye that is directly related to the amount of amplicon. Primers were derived from sequences in the 5' untranslated region (forward) and the second coding exon (reverse). The fluorescent probe, labeled with the FAM dye, contains sequence located within the first coding exon. When this exon is deleted, the probe cannot be cleaved, and no signal is generated. As a control for input amount, each cDNA sample was also amplified using primers and a probe, labeled with the VIC dye, for cyclophilin. The sequences of the primers and probes are as follows: ObR- probe 5' AAA CCACATAGAATTTCTGACACATCATCTTTC 3', forward primer 5' AGGAATCGTT CTGCAAATCCA 3', reverse primer 5' TATGCCAGGTTAAGTGCAGCTATC 3'; cyclophilin- probe 5' ACACGCCATAATGGCACTGGTGG 3', forward primer 5' TGT GCCAGGGTGGTGACTT 3', reverse primer 5' TCAAATTTCTCTCCGTAGATGGA CTT 3'. Data were analyzed with the ABI Sequence Detector® software. Every set of reactions contained a set of four serial two-fold dilutions of the same liver cDNA source, which was used to generate a standard curve for both ObR and cyclophilin. Amounts of

each transcript were calculated from the standard curve and levels of ObR were corrected for levels of cyclophilin (ObR level/ cyclophilin level).

Body composition analysis. Body composition was analyzed as previously described (Halaas et al. 1995). Carcasses were weighed and then oven dried in a 90° C oven until weight was constant. The total body water was calculated as the difference between the weights before and after drying. The carcass was then homogenized in a blender and duplicate aliquots were extracted with a soxhlet apparatus using a 3:1 mixture of chloroform:methanol. The extracted homogenate was dried overnight and weighed to calculate fat and lean mass.

Neuropeptide expression levels. Neuropeptide levels were quantitated by Taqman® real time PCR from individual hypothalami. Expression levels were measured in the obese $ObR^{SynI}KO$ mice, heterozygote controls, and in female ObR null mice. Primers and probes were made for AGRP, NPY, MCH, POMC, and CART. The sequences of the primers and probes, labeled with 5' FAM and 3' TAMRA, are as follows: AGRP- probe 5' TCCACAGAACCGCGAGTCTCGTTC 3', forward primer 5' CTTTGGCGGAGGTG CTAGA 3', reverse primer 5' GGACTCGTGCAGCCTTACACA 3'; NPY- probe 5' CA GAAAACGCCCCCAGAACAAGGC 3', forward primer 5' CACCAGACAGAGATAT GGCAAGA 3', reverse primer 5' TTTCATTTCCCATCACCACATG 3'; POMC- probe 5' CAGTGCCAGGACCTCACCACGGA 3', forward primer 5' TGCTTCAGACCTCC ATAGATGTGT 3', reverse primer 5' GGATGCAAGCCAGCAGGTT 3'; CART- probe 5' CACTGCGCACTGCTCCAGCG 3', forward primer 5' CGAGAAGAAGTACGGCC AAGTC 3', reverse primer 5' CCGATCCTGGCCCCTTT 3'; MCH- probe 5' AGGTGT

ATGCTGGGAAGAGTCTACCGACC 3', forward primer 5' GGAAGGAGAGATTTT GACATGCT 3', reverse primer 5' GCAGGTATCAGACTTGCCAACA 3'.

Neuroendocrine function. After sacrifice, mice were exsanguinated and blood was collected on EDTA. Plasma was collected after centrifugation and used for all assays. Leptin levels were determined using an ELISA kit from R&D Systems (Minneapolis, MN). Insulin levels were determined using an ELISA kit from Alpco Diagnostics (Windham, NH). Glucose levels were determined using the Trinder reagent from Sigma (St. Louis, MO). Triglyceride levels were determined using an enzymatic kit from Wako Diagnostics (Richmond, VA). Corticosterone, estradiol, testosterone, and thyroxine levels were determined using RIA kits from ICN (Costa Mesa, CA).

Liver triglyceride quantitation. 40-100 mg of liver from each mouse was homogenized in 4 ml of chloroform-methanol (2:1). 0.8 ml of 50mM NaCl was added to each sample. Samples were then centrifuged for 5 minutes at 3000 rpm. The lower phase was removed and duplicate 50 µl aliquots were evaporated under N_2 gas and then assayed for triglycerides using the Trinder reagent from Sigma (St. Louis, MO). Values are expressed as µg triglyceride / mg liver.

Statistical analysis. Data here, and throughout this thesis, are expressed as means plus or minus standard error of the mean (SEM). Unless otherwise indicated, significance was evaluated using the unpaired Student's *t* test. Significance of correlation coefficients was evaluated using the *t* test for correlation.

Materials and Methods from Chapter 4

Mice. Eight to ten week-old, female C57Bl/6J *ob/ob* or lean littermate mice (*ob/+* or +/+, referred to as wild-type) from the Jackson Laboratory (Bar Harbor, ME) were used for all expression studies and weight loss time courses.

RNA isolation. All mice were sacrificed in the middle of the light cycle and livers were removed and rapidly frozen in liquid nitrogen. Total RNA was prepared from frozen livers using Trizol reagent (Invitrogen, Carlsbad, CA).

Northern blotting. 10-20 μg of total RNA was separated by denaturing formaldehyde electrophoresis and transferred by capillary blot to positively charged nylon membranes as described (Ausubel et al. 1999). Blots were hybridized with $^{\alpha 32}$-P labeled PCR amplified cDNA probes. To control for equal loading, 18S RNA was stained with ethidium bromide or blots were hybridized with $^{\alpha 32}$-P labeled cyclophilin cDNA probes.

Time course. The time course experiment has been previously described (Soukas et al. 2000). All data presented in this thesis use material from this time course or an independent time course performed under identical conditions. Mice were individually caged and allowed to acclimate for a number of days prior to the start of the experiment. Alzet 2002 mini-osmotic pumps (Alza, Palo Alto, CA) were filled with PBS (free-fed and pair-fed groups) or 400 ng/ml leptin (Amgen, Thousand Oaks, CA), and implanted subcutaneously under anesthesia. RNA and plasma were isolated from *ob/ob* mice treated with subcutaneous leptin (infusion rate of 4.8 μg/24 hours via an Alzet miniosmotic pump) for 2, 4, and 12 days, untreated *ob/ob* mice, and free-fed *ob/ob* mice that received saline. As an additional control, RNA and plasma were isolated from saline-treated *ob/ob*

mice that were pair-fed to the leptin treated group also for 2, 4, and 12 days of treatment. The pair-fed group was staggered one day behind the other two groups and fed an amount equal to the average amount of food consumed by the leptin treated group over the previous 24 hours. Body mass and food intake were measured each morning. The body weight and food intake of these groups of mice is shown in Figure 4.2.

Liver histology and triglyceride quantitation. Liver histology was kindly performed by Dr. K. Sokol at the Rockefeller University Laboratory Animal Research Center. Livers were fixed in 4% paraformaldehyde, sectioned, and stained with hematoxylin and eosin. Liver triglyceride levels were determined by thin layer chromatography : gas chromatography (TLC:GC), as described below.

Microarrays and data analysis. RNA from the livers of these animals was prepared and hybridized to Affymetrix murine 6500 Gene Chips according to established methods (Affymetrix, Santa Clara, CA). Data were analyzed using a *K*-means clustering algorithm (Hartigan 1975; Kohonen 1997; Soukas et al. 2000). This computational method groups genes with similar patterns of expression over the time course experiment. For inclusion in the cluster analysis, a gene had to meet pre-established selection criteria, based on empirical observations from Northern blots. Specifically, genes were included that were given either an I (increased) or D (decreased) score by the Affymetrix GeneChip 3.1 software and that met one of the following cutoffs in at least two samples: (i) fold change ≥ 2.0 and average difference change ≥ 250, (ii) fold change ≥ 1.5 and average difference change ≥ 500, (iii) fold change $\geq \sim 1.5$ and average difference change ≥ 100.

Western blotting. Nuclear SREBP protein levels were measured as previously described (Soukas et al. 2000). Following treatment with saline, leptin, or saline with pair-feeding,

as described above, fresh livers were rinsed in ice cold saline supplemented with 1mM DTT and 0.5 mM PMSF. Livers were minced with a razor blade and homogenized with 10 strokes of a dounce homogenizer in NDS buffer (10 mM Tris at pH 7.5, 10 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol, 0.5 mM EGTA, 2 mM EDTA, 0.5% NP-40, 1 mM DTT, and 0.1 mM PMSF) supplemented with complete protease inhibitors at 4° C (Roche, Indianapolis, IN). The NP-40 concentration was raised to 1% and nuclei were pelleted by centrifugation at 700*g* for 5 minutes. Nuclei were then washed once with NDS buffer (1% NP-40), pelleted at 500*g* for 5 minutes, resuspended in 1 volume of 1% citric acid, lysed (by the addition of 2.5 volumes of 0.1 M Tris, 2.5% SDS, and 0.1 M DTT), sonicated, and heated to 90°C for 5 min. Western blotting was performed using nuclear extract, according to previously established methods (Sambrook et al. 1989). SREBP was detected using 5g/ml rabbit anti-mouse SREBP-1 (kindly provided by M. Brown, University of Texas Southwestern Medical Center, Dallas, TX). Bound antibodies were detected using an HRP-conjugated anti-rabbit IgG secondary antibody (Organon Teknika, West Chester, PA) and visualized using enhanced chemiluminescence plus reagents (Amersham, Buckinghamshire, England).

Statistical analysis. Data are expressed as means plus or minus SEM. Significance was evaluated using the unpaired Student's *t* test.

Materials and Methods from Chapter 5

Identifying and ranking leptin-regulated genes. In order to prioritize leptin-regulated genes for detailed functional analysis, we developed an algorithm to identify and rank

genes that are specifically repressed by leptin. This algorithm ranked genes based on the extent to which their expression was (1) increased in *ob/ob* liver compared to wild type (2) repressed by leptin treatment, and (3) different between leptin treatment and pair-feeding. Specifically, we selected genes, which met the above criteria for cluster analysis, whose expression was increased in *ob/ob* relative to wild-type and corrected by leptin administration. The selected genes were then scored using the following two criteria: (1) the magnitude of the difference in expression between the untreated *ob/ob* sample and the day 12 leptin treated sample and (2) the magnitude of the distance between the leptin expression profile and the pair-fed expression profile. The above distance was defined to be the Euclidean distance between the normalized 3-dimensional vectors composed of the 2, 4, and 12 day leptin-treated and pair-fed profiles. To combine the two scores, the genes were ranked according to both parameters and then the pair of ranks was averaged for each gene. This final average rank served as the score for each gene. Genes with the lowest scores are those most strongly leptin-regulated.

Northern blotting. Membranes were hybridized with $^{\alpha 32}$-P labeled cDNA probes made using the untranslated region of SCD-1, which specifically hybridizes to SCD-1 and not SCD-2 (Ntambi et al. 1988; Kaestner et al. 1989). To control for equal loading a cDNA probe for the small mitochondrial RNA pAL-15 was used.

SCD activity assay. These assays measure the conversion of [1-^{14}C] stearoyl-CoA to [1-^{14}C] oleate, as previously described (Miyazaki et al. 2000). Tissues were homogenized in 10 volumes of buffer A (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, 1 mM PMSF, pH 7.4). The microsomal fractions (100,00 x *g*) were isolated by sequential centrifugation. Reactions were performed at 37°C for 5 min with 100 μg protein

homogenate and 6 μM of [1-^{14}C] stearoyl-CoA (60,000 rpm), 2 mm NADH, 0.1 M Tris-HCl, pH 7.2. After the reaction, fatty acids were extracted and methylated with 10% acetic chloride/methanol. Saturated and monounsaturated fatty acid methyl esters were separated by 10% $AgNO_3$-impregnated TLC using hexane:diethyl ether (9:1) as developing solution. The plates were sprayed with 0.2% 2',7'-dichloroflourescein in 95% ethanol, and the lipids were identified under UV light. Fractions were scraped off of the plate and radioactivity was measured using a liquid scintillation counter.

Lipid Analysis. Total lipids were extracted from liver with Folch solvent (chloroform/methanol 2:1). At the start of the liver extraction, internal standards of C17:0 triglyceride, cholesteryl ester, and phosphatidyl choline were added to quantitate each lipid class (Nu Check Prep, Elysian, MN and Sigma, St. Louis, MO). The lipid classes in the liver extract were separated by thin layer chromatography using silica gel G and a developing solvent of hexane/diethyl ether/acetic acid (60:40:1). The fatty acids in each lipid class were methylated with fresh 5% methanolic HCl and analyzed with a gas chromatograph (model 5890, Hewlett-Packard, Palo Alto, CA) equipped with a 100m x 0.25mm SP2560 fused silica capillary column (Supelco, Bellefonte, PA).

Mouse crosses. Asebia (ab^J/ab^J) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). These mice have a spontaneous deletion of the first four exons of the SCD-1 gene and express no SCD-1 RNA or protein (Gates and Karasek 1965; Zheng et al. 1999). Double mutant ab^J/ab^J; *ob/ob* mice were generated by two successive crosses. First, ab^J/ab^J mice (mutation arose spontaneously in Balb/cJ mice) (more detailed background strain information is available on the internet at http://www.informatics.jax.org/external/ festing/mouse/docs/ABJ.shtml) were crossed to

ob/+ (C57Bl/6 background). Then, progeny were intercrossed to generate the following groups of mice: (1) double mutant mice (*ab^J/ab^J; ob/ob*), (2) *ob/ob* controls (*$ab^J/+$; ob/ob* and *+/+; ob/ob*), (3) asebia mice (*ab^J/ab^J; ob/+* and *ab^J/ab^J; +/+*), and (4) controls (*$ab^J/+$; ob/+*, *$ab^J/+$; +/+*, *+/+; ob/+*, and *+/+; +/+*). *ob* genotypes were determined by PCR amplifying genomic DNA using the forward primer 5' GACTTCATTCCTGGG CTTCA 3' and the reverse primer 5' TCTGTGGAGTAGAGTGAGGC 3' and the following cycling conditions: 94°C 5 min, 40 x (94°C 1 min, 55°C 1 min, 68°C 2 min), 68°C 7 min. The 247 bp PCR product was then digested with DdeI (a DdeI site is added by the *ob* mutation) and run on a 3% agarose gel. The wild-type allele shows a 152 bp and 32 bp band and the *ob* allele shows 105 bp, 47 bp, and 32 bp bands. For practical purposes only the 152 bp and 105 bp bands were used to determine genotypes. *ab* genotypes were determined by Southern blotting of NcoI digested genomic DNA. Blots were hybridized using an $^{\alpha 32}$-P labeled SCD-1 exon 5 probe, PCR amplified from cDNA using the forward primer 5' TGTGCTTCATCCTGCCCACG 3' and the reverse primer 5' GATTGAATGTTCTTGTCGTAGG 3' and the following cycling conditions: 94°C 5 min, 35 x (94°C 30 sec, 60°C 30 sec, 72°C 1 min), 72°C 7 min. *ab/ab*, *ab/+*, and *+/+* genotypes showed characteristic banding patterns.

Carcass analysis. Carcass analysis was done as described above.

Food intake. 12-18 week old were individually caged and allowed to acclimate for 1 week. 24-hour food consumption was measured over 8 consecutive days, and these values were averaged for the mice in each group. Food intake for males and females was indistinguishable and was therefore pooled.

Oxygen consumption. 14-16 week old mice were placed in an Oxymax indirect calorimeter (Columbus Instruments, Columbus, OH) and allowed 2 hours to acclimate. The calorimeter was calibrated and operated according to the manufacturer's instructions. Measurements were taken for 5 hours during the middle of the light cycle. The first reading was discarded, and the total V_{O2} was determined by averaging the other 19 readings. The resting V_{O2} is the average of all readings that are one standard deviation below the total V_{O2}, as these readings represent periods of inactivity.

Plasma assays. Leptin levels were measured by ELISA (R&D Systems, Minneapolis, MN). β–hydroxybutyrate levels were measured using an enzymatic assay (Sigma, St. Louis, MO).

Liver histology and triglyceride quantitation. Liver histology was kindly performed by Dr. T. Scase at the Bobst Hospital of the Animal Medical Center (New York, NY). Livers were fixed in 4% paraformaldehyde, sectioned, and stained with hematoxylin and eosin. Liver triglyceride levels were determined as described above, with a monor modification: one drop of Triton X-100 was added to each aliquot prior to evaporating under N_2 gas.

VLDL production assay. Mice were fasted for five hours prior to injection with tyloxapol (Triton-1339) (Sigma, St. Louis, MO) as previously described (Merkel et al. 1998). Mice were injected via the tail vein with 0.5 mg/kg tyloxapol dissolved in saline at a concentration of 0.15 g/ml. Tail bleeds were done just before injection (t0) and 45 and 90 minutes following injection. Plasma triglycerides were assayed using an enzymatic reagent (Sigma, St. Louis, MO). The slope of the line denotes the rate of VLDL production.

Fatty Acyl CoA Levels. Mice had their food removed at the beginning of the light cycle and were studied 6-10 hours later. Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine. Livers were freeze-clamped in situ using tongs that had been cooled to –70°C and were stored at –80°C. Tissues were homogenized and long chain fatty acids were extracted and analyzed by LC/MS/MS analysis using a tandem mass spectrometer (Applied Biosytems, Foster City, CA) as previously described (Neschen et al. 2002).

Statistical Analysis. Data are expressed as means plus or minus SEM. Significance was evaluated using the unpaired Student's *t* test.

Materials and Methods from Chapter 6

Alcohol Studies. Group housed, 5-7 week old male and female ab^J/ab^J and littermate control mice ($ab^J/+$ or +/+) were acclimated to alcohol by the introduction of a 10% ethanol solution in addition to regular drinking water. After 1 week (time 0), regular drinking water was removed and mice were only given 10% ethanol to drink. Groups of mice were sacrificed after 3 and 5 weeks of ethanol consumption. As a control, non-alcohol fed, age-matched ab^J/ab^J and littermate control mice ($ab^J/+$ or +/+) were also studied. To measure food intake and alcohol consumption, 8-10 week old ab^J/ab^J and littermate control mice ($ab^J/+$ or +/+) were individually caged, and food and alcohol intake were recorded weekly. Total caloric intake was based on a caloric value of 5.5251 kcal/ml for pure ethanol and 4 kcal/g for mouse chow.

Lipodystrophy Studies. Double mutant ab^J/ab^J;*aP2-SREBP-1c* transgenic mice were generated by two successive crosses. First, female ab^J/ab^J mice (strain information

described above) were crossed to male *aP2-SREBP-1c* transgenic mice obtained from the Jackson Laboratory (Bar Harbor, ME) (C57Bl6/J x SJL strain). Then ab^J/+;*aP2-SREBP-1c* transgenic male progeny were crossed to ab^J/+ female progeny to generate the following groups of mice: (1) double mutant mice (ab^J/ab^J;*aP2-SREBP-1c tg*), (2) *aP2-SREBP-1c tg* controls (ab^J/+;*aP2-SREBP-1c tg* and +/+;*aP2-SREBP-1c tg*), (3) asebia mice (ab^J/ab^J), and (4) controls (ab^J/+ and +/+). Genotyping was performed by genomic PCR for the transgene and by Southern blotting for SCD-1.

Liver histology and triglyceride quantitation. Liver histology was performed at the Bobst Hospital of the Animal Medical Center (New York, NY). Livers were fixed in 4% paraformaldehyde, sectioned, and stained with hematoxylin and eosin. Liver triglyceride levels were determined as described above, except for the additional step of adding one drop of Triton X-100 to each aliquot prior to evaporating under N_2 gas.

Statistical Analysis. Data are expressed as means plus or minus SE. Significance was evaluated using the unpaired Student's *t* test.

Materials and Methods from Chapter 7

Northern Blotting. C57Bl/6J, *ob/ob*, db^{3J}/db^{3J}, db^{Kls}/db^{Kls}, NZO, A^y, and AKR/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The AKR/J mice (strain is susceptible to dietary obesity) were not diet-induced. *ob/*ob *tg* mice were maintained at the Rockefeller University Laboratory Animal Research Center (Ioffe et al. 1998). All mice were 8-12 weeks old. Liver RNA was isolated from 2-3 animals of each genotype, sacrificed in the fed state or after a 48 hour fast. IGFBP-2 RNA levels were determined by Northern blotting, as described above, using an $^{\alpha 32}$-P labeled PCR amplified IGFBP-2

cDNA probe. Equal loading was confirmed by ethidium bromide staining of 18S RNA or by hybridization with a cyclophilin probe.

Mice. *IGFBP-2$^{-/-}$;ob/ob* mice were generated by two successive crosses. *ob/+* mice (C57Bl/6J strain) obtained from the Jackson Laboratory (Bar Harbor, ME) were crossed to *IGFBP-2$^{-/-}$* mice (129/ReJ x C57Bl6/J) generated and maintained at the Robert Wood Johnson Medical School / University of Medicine and Dentistry New Jersey (Piscataway, New Jersey) (Wood et al. 2000). *IGFBP-2$^{+/-}$;ob/+* progeny were intercrossed to generate a line of *IGFBP-2$^{-/-}$;ob/+* mice. These mice were intercrossed to generate *IGFBP-2$^{-/-}$;ob/ob* mice. *ob/ob* controls (C57Bl6/J strain) were obtained from the Jackson Laboratory (Bar Harbor, ME).

Leptin Administration. Leptin treatment was performed as described above for Chapter 4. Mice were individually caged and allowed to acclimate for more than a week prior to the start of the experiment. Alzet 2002 mini-osmotic pumps (Alza, Palo Alto, CA) were filled with PBS (saline group) or 400 ng/ml leptin (Amgen, Thousand Oaks, CA), and implanted subcutaneously under anesthesia. Groups of *ob/ob* and *IGFBP-2$^{-/-}$;ob/ob* mice were treated with subcutaneous leptin (infusion rate of 4.8 μg/24 hours via an Alzet miniosmotic pump) or PBS for 10 days. For *ob/ob* mice, n = 4 for saline (all males, age at start of experiment = 76 days) and n = 6 for leptin (4 females and 2 males, average age at start of experiment = 66 ± 3.2 days). For IGFBP-2$^{-/-}$;*ob/ob* mice, n = 4 for saline (all females, average age at start of experiment = 103.8 ± 8.6 days) and n = 5 for leptin (all females, average age at start of experiment = 103.6 ± 8.3 days).

Plasma Assays. Leptin levels were measured by ELISA (R&D Systems, Minneapolis, MN). Glucose levels were measured using an enzymatic assay (Sigma, St. Louis, MO).

Statistical Analysis. Data are expressed as means plus or minus SEM. Significance was evaluated using the unpaired Student's *t* test.

Chapter 3: Selective Deletion of Leptin Receptor in Neurons Leads to Obesity

Introduction

Leptin is an adipocyte hormone that functions as an afferent signal in a negative feedback loop that regulates energy balance (Zhang et al. 1994; Friedman and Halaas 1998; Spiegelman and Flier 2001). Mice with mutations in leptin (*ob/ob*) or its receptor *(db/db*) are hyperphagic and severely obese (Tartaglia et al. 1995; Chen et al. 1996b; Lee et al. 1996). These mutant mice also manifest a large number of metabolic and endocrine abnormalities including diabetes, hypercortisolemia, infertility, and cold intolerance (Coleman 1978; Coleman 1982). *ob/ob* and *db/db* mice also have enlarged, steatotic livers (Yen et al. 1976; Koteish and Diehl 2001). The fatty liver is in part a result of an increased rate of hepatic lipogenesis, which is also thought to contribute to the development of obesity (Kaplan and Leveille 1981).

Treatment of *ob/ob* mice with leptin reduces food intake and body weight and corrects the metabolic and endocrine defects associated with leptin deficiency. Leptin treatment also normalizes the hepatomegaly and associated elevations in hepatic glycogen and lipid (Levin et al. 1996). Infusions of leptin into wild type mice at physiological amounts results in a dose dependent reduction in food intake and body weight (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995). This metabolic response to leptin in both *ob/ob* and wild type mice is novel and is not solely a consequence of its anorectic effects (Soukas et al. 2000). Intracerebroventricular leptin

has similar effects, but at much lower doses, suggesting that leptin has direct effects on brain (Campfield et al. 1995; Halaas et al. 1997).

The leptin receptor, which has five splice variants (ObRa-e), is broadly expressed, however, and the relative importance of leptin's effects on brain vs. peripheral sites is unclear (Ghilardi et al. 1996; Fei et al. 1997). This distinction is of importance because leptin deficiency is associated with myriad abnormalities in many tissues including liver. While leptin has been suggested to act directly to deplete triglycerides, the contribution of these actions to body weight homeostasis, deposition of lipid in peripheral sites, and neuroendocrine function *in vivo* is untested (Shimabukuro et al. 1997a; Minokoshi et al. 2002). In order to determine the role of leptin action in the central nervous system and the periphery, we are systematically deleting ObR in a tissue-specific fashion using the Cre-loxP system. Here we present data from mice with either neuronal ($ObR^{SynI}KO$) or hepatocyte-specific ($ObR^{Alb}KO$) deletions of ObR.

Among the $ObR^{SynI}KO$ mice, the extent of obesity was negatively correlated with the level of ObR in hypothalamus and those animals with the lowest levels of ObR exhibited an obese phenotype. The obese mice with low levels of hypothalamic ObR also show elevated plasma levels of leptin, glucose, insulin, and corticosterone. The hypothalamic levels of AGRP and NPY are increased in these mice. These data indicate that leptin has direct effects on neurons and that a significant proportion, or perhaps the majority, of its weight reducing effects are the result of its actions on brain. To explore possible direct effects of leptin on a peripheral tissue, we also characterized $ObR^{Alb}KO$ mice. These mice weigh the same as controls and have no alterations in body composition. Moreover, while *db/db* mice and $ObR^{SynI}KO$ mice have enlarged fatty

livers, $ObR^{Alb}KO$ mice do not. In summary, these data suggest that the brain is a direct target for the weight reducing and neuroendocrine effects of leptin and that the liver abnormalities of *ob/ob* and *db/db* mice are secondary to defective leptin signaling in the brain.

Results

Generation of tissue-specific knockout mice

We have used the Cre-loxP system to generate mice with tissue specific deletions of ObR (Gu et al. 1994). The construction of the targeting plasmid is described in Chapter 2 (Materials and Methods) (Figure 3.1a). The first coding exon of ObR was flanked by loxP sites. This exon contains the signal sequence and deletion of it by Cre-mediated recombination was predicted to inactivate all splice variants. Following transfection into mouse embryonic stem (ES) cells, 3/72 G418-resistant clones had undergone homologous recombination (Figure 3.1b). The targeting cassette was deleted by transiently transfecting one of the homologous recombinant ES cell lines with a plasmid expressing Cre recombinase and screening for clones with a type II deletion (Figure 3.1a). Clones with the correct genotype, $ObR^{flox/+}$, were confirmed by Southern blotting and injected into C57Bl/6 blastocysts (Figure 3.1c). Breeding of chimeras confirmed that animals with a germline insertion of loxP sites into ObR had been generated. Mice homozygous for the floxed allele, $ObR^{flox/flox}$, were generated by crossing $ObR^{flox/+}$ animals.

$ObR^{flox/flox}$ mice were viable, fertile, and indistinguishable from wild-type or $ObR^{flox/+}$ mice indicating that the insertion of loxP sites into the introns flanking the first

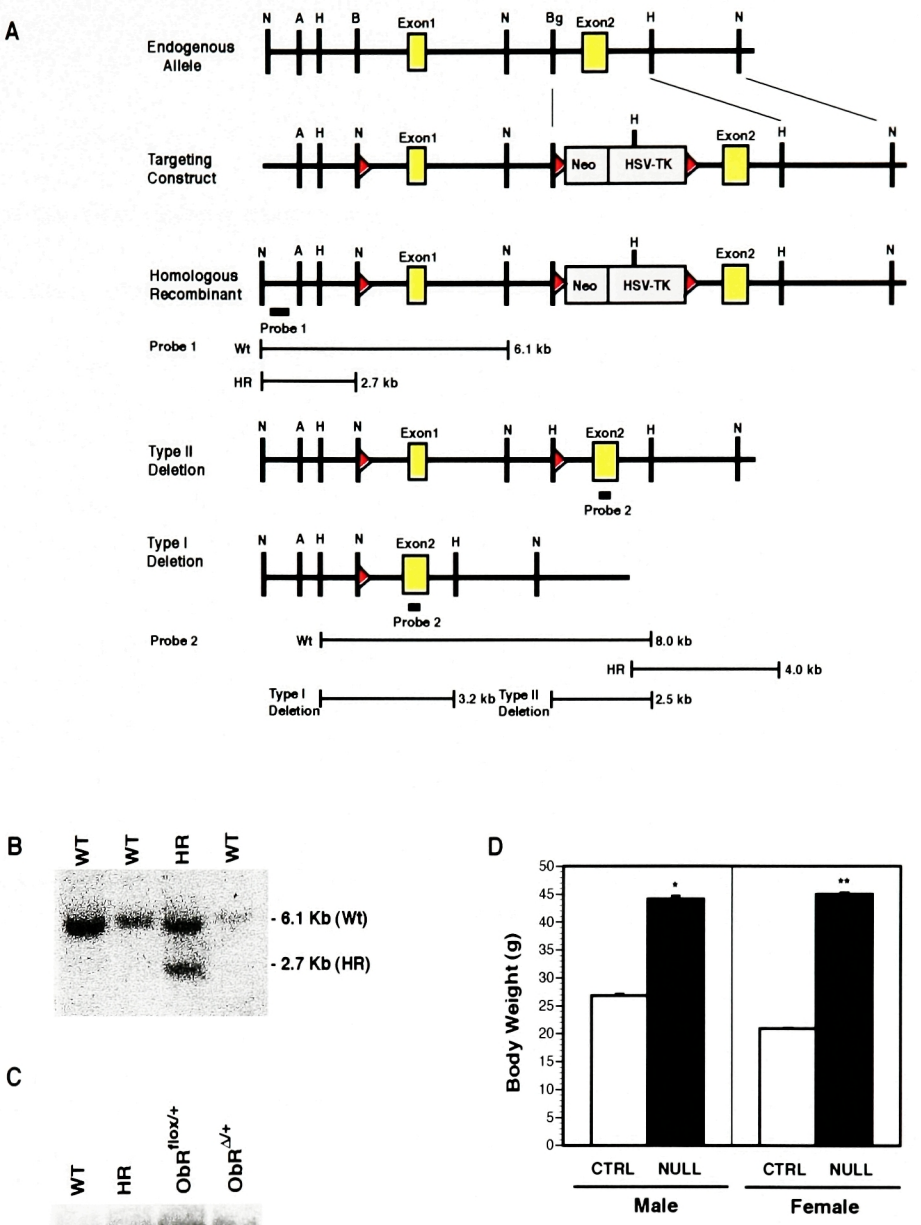


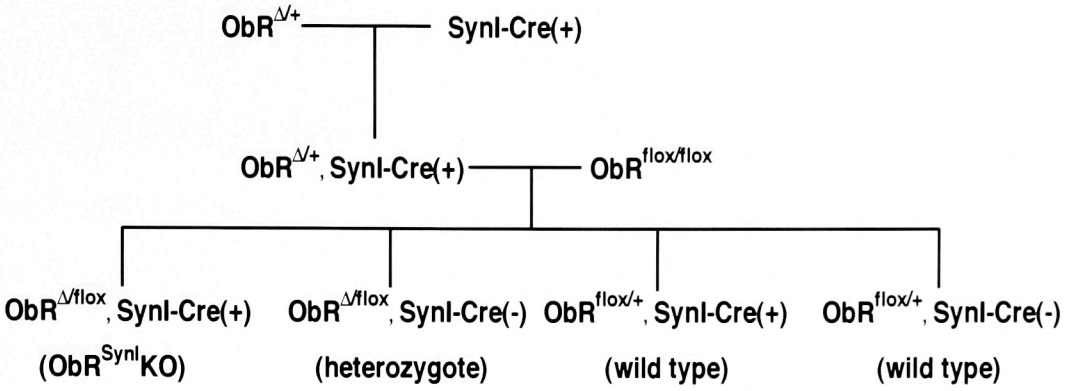
Figure 3.1 LoxP targeting of the ObR locus. Gene targeting was used to insert loxP sites on either side of the first coding exon of ObR. (A) Restriction maps (from top to bottom) of the genomic locus, targeting vector, homologous recombinant, and the type II and type I deletion alleles. Probe 1, located outside the targeting construct, was used to screen for homologous recombinants. Probe 2, located in the second coding exon, distinguishes the endogenous allele, homologous recombinants, and type I (deletion of the first coding exon) and II (deletion of the targeting cassette leaving loxP sites on either side of the first coding exon) deletions. A, AflII; B, BamHI; Bg, BglII; H, HindIII; N, NcoI. (B) Southern blot analysis of NcoI-digested genomic DNA from ES cell clones using Probe 1. The endogenous allele (Wt) and homologous recombinants (HR) migrated at the predicted sizes. (C) Southern blot analysis of Hind-III digested genomic DNA using Probe 2. The endogenous allele (Wt), homologous recombinant (HR) and Type II deletion were detected from ES cell DNA. $ObR^{flox/+}$ mice were generated from the Type II deletion and crossed to adenovirus EIIA-cre mice. The Type I deletion was detected in tail DNA from progeny derived from this cross. All alleles migrated at the predicted sizes. (D) Body weight at 8 weeks of age of $ObR^{\Delta/\Delta}$ and littermate control ($ObR^{\Delta/+}$) mice. Data represent the mean +/- SEM of at least 9 animals of each genotype and gender. *, $p < 0.02$, **, $p < 0.001$ in an unpaired Student's t test.

coding exon did not interfere with ObR function. To determine whether Cre-mediated deletion of the first coding exon results in a null allele, $ObR^{flox/+}$ mice were crossed with transgenic mice expressing Cre at an early embryonic stage (Lasko et al. 1996). Mice with the genotype $ObR^{\Delta/+}$, heterozygous for a germline deletion of the first coding exon (Type I deletion), were generated and mated to produce homozygous $ObR^{\Delta/\Delta}$ mice, also referred to as ObR null mice (Figure 3.1c). ObR null mice are massively obese and indistinguishable from db^{3J}/db^{3J} mice, which are also null for all ObR isoforms (Figure 3.1d) (Lee et al. 1997). These data confirm that the floxed allele is wild type and that the deleted allele is null, thus validating the targeting strategy.

Neuron-specific ObR knockout

Neuron-specific ObR knockout mice, designated $ObR^{SynI}KO$ were generated by two successive crosses (Figure 3.2a, and described in Materials and Methods). These mice carry one floxed and one null allele so that Cre-mediated inactivation of only one allele, rather than two, is sufficient to delete ObR in a given cell. The $ObR^{SynI}KO$ mice were compared to littermate controls with the genotype $ObR^{\Delta/flox}$, SynI-Cre(-), also referred to as heterozygotes. These mice were used as controls because some phenotypic changes have been observed in *db/+* mice (Coleman 1979). Data were also obtained from lean littermates with the genotypes $ObR^{flox/+}$, SynI-Cre (+) or $ObR^{flox/+}$, SynI-Cre (-), both of which are referred to as wild type. Genotypes of all mice were determined using PCR and Southern blotting.

A



B

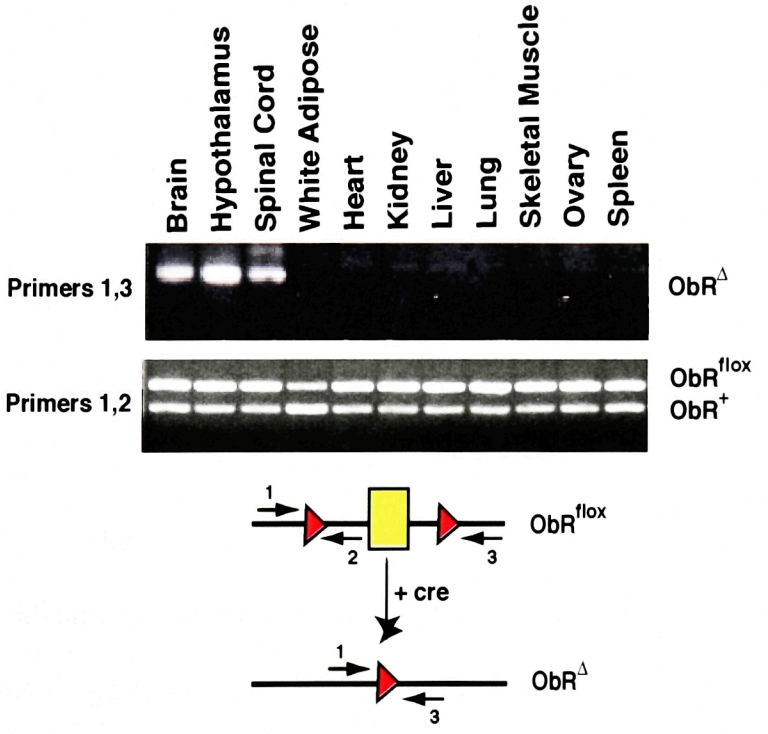


Figure 3.2 Cre-mediated recombination specifically in the brain of ObR^{SynI}KO mice.
(A) The breeding strategy that was used to generate ObR^{SynI}KO mice and littermate controls is shown. ObR^{Alb}KO mice (described below) were generated using the same strategy.
(B) Genomic DNA was prepared from several tissues from ObR$^{flox/+}$, SynI-Cre(+) mice and PCR amplified using primers flanking the first coding exon of ObR. The locations of the primers are shown on the schematic below the gel. In tissues expressing the Cre recombinase, exon 1 is excised and a single loxP site remains, generating an ObR$^{\Delta}$ allele. While primers 1 and 3 can amplify a product from the ObR$^{\Delta}$ allele, in the ObR^{flox} or ObR^{+} (wild type) DNA, the primers are separated by a distance too great for amplification to occur. As a control, primers 1 and 2 were used to amplify both the ObR^{flox} allele and the ObR^{+} alleles from all tissues. The ObR^{+} allele produces a slightly smaller product due to the absence of loxP sequences.

In the synapsinI-Cre transgenic line, expression of Cre recombinase is controlled by the rat synapsin I promoter. This promoter has been shown to drive Cre expression specifically in neurons (Hoesche et al. 1993). Crossing SynI-Cre^{+} mice to LacZ indicator transgenic strains showed that Cre activity was first detectable at E12.5 and restricted to brain, spinal cord, and the dorsal root ganglion, and absent from astrocytes and glia (Zhu et al. 2001). To confirm the tissue specificity of Cre-mediated recombination, a qualitative PCR assay was developed that was capable of detecting recombination between the lox sites flanking the first coding exon of ObR in genomic DNA. In ObR$^{flox/+}$ synapsinI-Cre (+) mice, Cre-mediated recombination was restricted to brain, hypothalamus and spinal cord (Figure 3.2b). As previously indicated by reporter gene expression in SynI-CAT transgenic mice, low level recombination was also seen in testis (data not shown).

The weight of the ObR^{SynI}KO mice was compared to that of heterozygous mice at four months of age. While the weights of ObR^{SynI}KO mice, on average, were no different than heterozygotes, in both sexes a subset of the ObR^{SynI}KO mice had an increased body weight (Figure 3.3a). The percent body fat of these mice (shown in yellow) was more than 3 standard deviations greater than the average percent body fat of heterozygotes (Figure 3.3a). This suggested the possibility that the extent of the knockout of ObR was variable and that the most obese ObR^{SynI}KO animals had the lowest levels of ObR. In order to assess the efficiency of Cre-mediated recombination, real time PCR assays (Taqman®) were performed to quantitate ObR RNA using cDNA prepared from individual hypothalami (Livak et al. 1995). Previous data have suggested that in neurons,

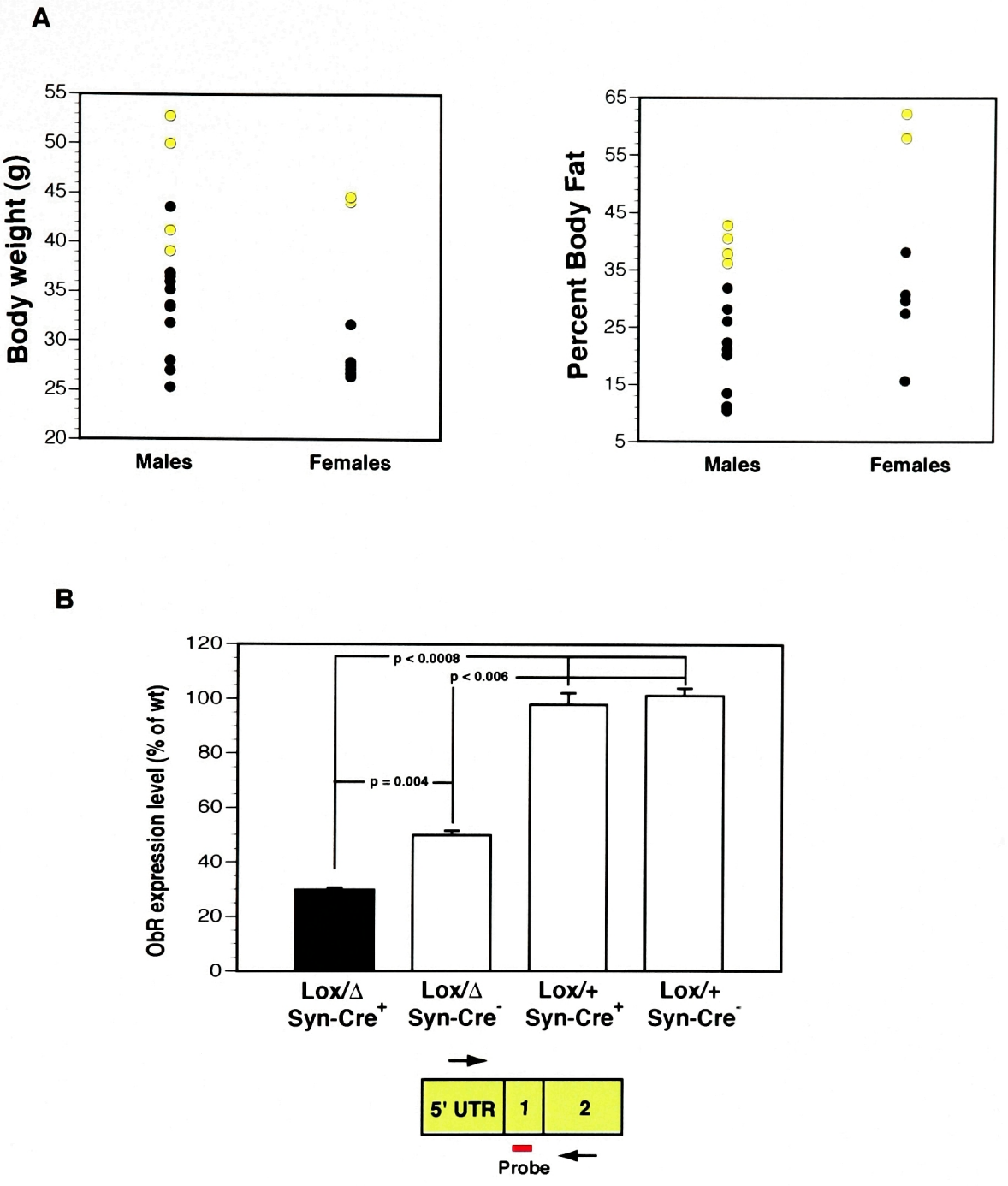


Figure 3.3 ObR RNA levels. (A) The distribution of body weight and percent body fat for male and female $ObR^{SynI}KO$ mice at 16 weeks of age is shown. Those mice with less than 15% of ObR RNA (as identified in Figure 3B) are shown in yellow. (B) Expression levels were determined using Taqman® real time PCR with the ABI Prism® 7700 Sequence Detection System. The locations of primers and fluorescent probe are as indicated. Primers were derived from sequences in the 5' untranslated region and the second coding exon. The fluorescent probe is located within the first coding exon 1. When this exon is deleted, no signal is generated. As a control for input amount, each cDNA sample was also amplified using primers and a probe for cyclophilin. Data were analyzed with ABI Sequence Detector® software and the levels of ObR were normalized to cyclophilin. Levels are represented as the percentage of the levels in $ObR^{flox/+}$, SynI-Cre(+) and $ObR^{flox/+}$, SynI-Cre(-) wild type mice. Data represent the mean +/- SEM. At least 13 animals were analyzed for each genotype. p values for comparisons between genotypes are indicated.

ObR is expressed at highest levels in hypothalamus and at much lower levels in other brain regions. This assay detected all forms of the leptin receptor. $ObR^{flox/+}$, SynI-Cre(+) and $ObR^{flox/+}$, SynI-Cre(-) mice had equivalent levels of ObR expression, and thus both genotypes were designated as wild-type (Figure 3.3b). As expected, $ObR^{\Delta/flox}$, SynI-Cre (-) mice, which have one allele inactivated in the germ line, have 50% as much RNA as wild type mice ($p < 0.006$). The average level of ObR RNA was significantly lower in $ObR^{SynI}KO$ mice than in $ObR^{\Delta/flox}$, SynI-Cre (-) heterozygote littermates (30% vs. 50% of wild type respectively, $p = 0.004$). Moreover, while many of the $ObR^{SynI}KO$ mice had levels of ObR RNA that were indistinguishable from heterozygotes, a subset of the $ObR^{SynI}KO$ mice had markedly reduced levels of ObR. These results confirmed that Cre-mediated recombination is variable, and indicated that in many animals there is no evident recombination.

We next considered whether the most obese $ObR^{SynI}KO$ animals (i.e. percent body fat 2.5 standard deviations greater than heterozygotes) had lower levels of ObR RNA than the lighter ones. Analysis of the data indicated that percent body fat and body weight were significantly increased in each of six animals in which ObR RNA was less than 15% that of wild-type (shown in yellow in Figure 3.3a) ($p < 0.005$ for percent body fat, $p < 0.05$ at all ages greater than 5 weeks for body weight). This confirmed that a significant deletion of ObR is associated with an obese phenotype. Furthermore, in those cases where the deletion is most extreme, a severely obese phenotype is evident. Two $ObR^{SynI}KO$ females and one $ObR^{SynI}KO$ male had a greater than 97% reduction of ObR RNA and each weighed more than 50 grams at sacrifice, weights that approach, but do

not equal, that of *db/db* mice. In these studies, as well as studies of the $ObR^{Alb}KO$ mice (see below), there were no lean mice with hypothalamic ObR RNA levels less than 15% that of wild type, and there were no obese mice (obese defined as percent body fat > 2.5 SD above heterozygotes: 30.5% for males, 47.8% for females) with RNA levels greater than 15% that of wild type. These data suggest that a near normal body weight can be maintained until markedly reduced levels of ObR in hypothalamus are evident. In all following studies, the phenotype of the animals with <15% wild type levels of hypothalamic ObR RNA was compared to heterozygotes. These mice are referred to as obese $ObR^{SynI}KO$ mice.

The weights of the obese $ObR^{SynI}KO$ mice were significantly increased relative to heterozygotes at all time points greater than 5 weeks of age ($p < 0.05$). At 5 months of age, males and females weighed 35% and 66% more than heterozygotes respectively. (Figure 3.4a). The increase in body weight was associated with increased adipose tissue mass and, as is also the case in *ob/ob* and *db/db* mice, decreased lean body mass (Table 3.1). At sacrifice, the percent body fat of $ObR^{SynI}KO$ mice was 39% in males and 60% in females, compared to 15% in male heterozygotes and 26% in female heterozygotes.

The obese $ObR^{SynI}KO$ mice were also analyzed with respect to a number of other abnormalities associated with the *db/db* mutation (Table 3.1). Plasma leptin concentrations were elevated 5.8–fold in obese $ObR^{SynI}KO$ males ($p < 0.05$) and 8.3–fold in obese $ObR^{SynI}KO$ females ($p < 0.0001$) and were highly correlated with percent body fat ($r = 0.82$, $p < 0.01$, *t* test for correlation). In addition, plasma insulin was increased 6–fold in obese $ObR^{SynI}KO$ males ($p < 0.05$) and 16.1–fold in $ObR^{SynI}KO$ females. Glucose was increased 2.3-fold in $ObR^{SynI}KO$ females ($p < 0.005$), and was unchanged in

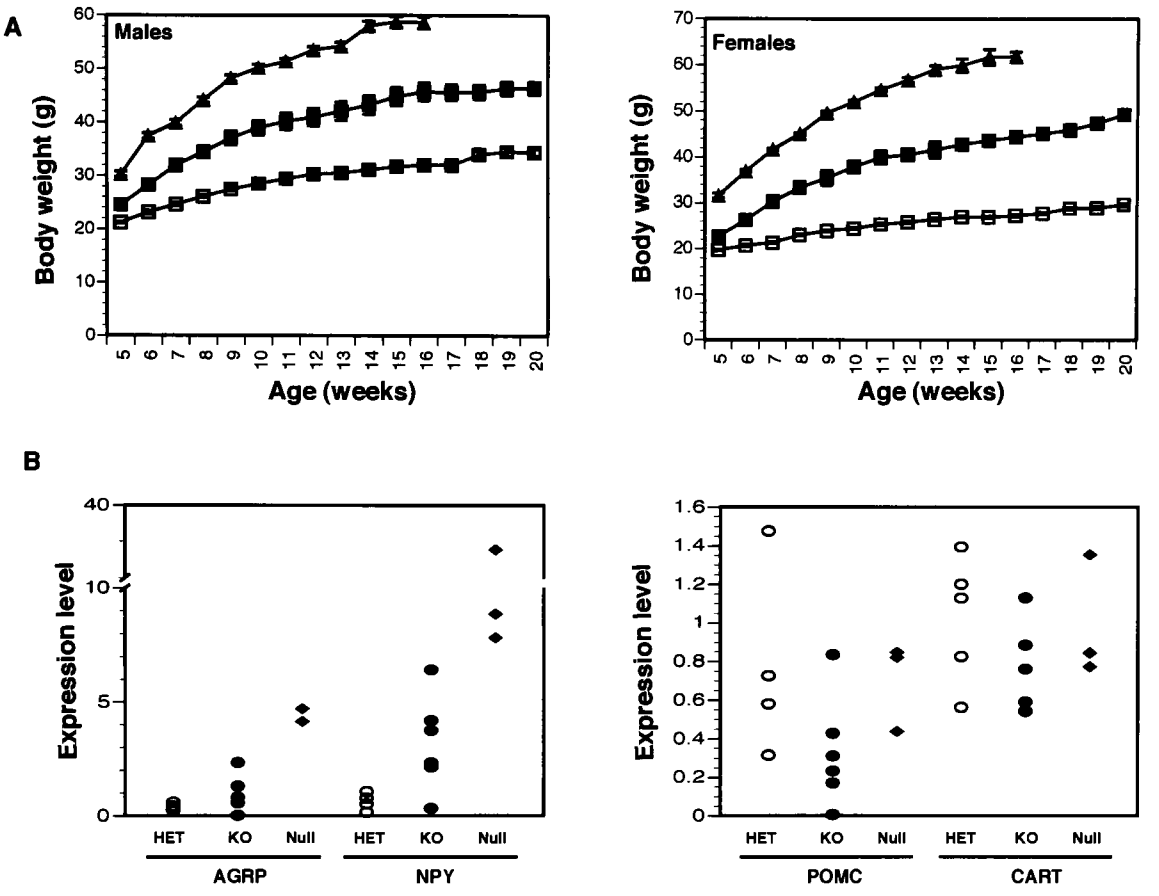


Figure 3.4 Obesity in a subset of ObR^{SynI}KO mice. Significant obesity was evident in those ObR^{SynI}KO mice that had 15% or less ObR RNA in the hypothalamus than wild type mice. (A) Weight curves of male and female ObR^{SynI}KO mice (solid squares), heterozygote littermates (open squares), and ObR null mice (solid triangles). Mice were weighed weekly from 5 weeks of age. Data represent the mean +/- SEM of 4 male and 2 female ObR^{SynI}KO mice and 7 male and 9 female heterozygote littermates. For both sexes, ObR^{SynI}KO vs. heterozygotes, $p < 0.05$ at all ages and ObR^{SynI}KO vs. ObR null, $p < 0.05$ at all ages. (B) Expression levels of AGRP, NPY, POMC, and CART were determined by Taqman ® and are expressed as normalized to cyclophilin. Levels were measured in ObR^{SynI}KO, heterozygotes, and ObR nulls. n = 5 for heterozygotes, n = 6 for knockouts, and n = 3 for null mice.

Body Composition and Neuroendocrine Function in $ObR^{SynI}KO$ and $ObR^{Alb}KO$ Mice

	$ObR^{SynI}KO$	$ObR^{SynI}Het$	$ObR^{Alb}KO$	$ObR^{Alb}Het$
Percent Fat Mass				
Males	39.4 +/- 0.7 (4)	15.4 +/- 0.8 (8)^{A}	14.0 +/- 1.5 (4)	13.2 +/- 0.5 (5)
Females	60.1 +/- 1.5 (2)	25.8 +/- 0.9 (10)^{A}	15.4 +/- 0.9 (7)	15.6 +/- 1.1 (5)
Percent Lean Mass				
Males	19.7 +/- 0.6 (4)	25.7 +/- 0.2 (8)^{A}	30.2 +/- 0.8 (4)	30.2 +/- 0.8 (5)
Females	12.7 +/- 1.2 (2)	22.6 +/- 0.3 (10)^{A}	28.0 +/- 0.7 (4)	27.5 +/- 0.7 (5)
Percent Water Mass				
Males	40.9 +/- 0.3 (4)	59.0 +/- 0.6 (8)^{A}	55.8 +/- 1.3 (4)	56.6 +/- 0.6 (5)
Females	27.2 +/- 0.3 (2)	51.6 +/- 0.6 (10)^{A}	56.6 +/- 0.9 (4)	56.9 +/- 0.7 (5)
Leptin (ng/mL)				
Males	73.8 +/- 8.5 (4)	12.7 +/- 0.4 (10)^{B}	13.1 +/- 0.8 (9)	13.9 +/- 0.7 (15)
Females	125.0 +/- 0.7 (2)	15.1 +/- 0.8 (12)^{A}	12.7 +/- 0.9 (6)	6.7 +/- 0.2 (12)^{B}
Insulin (ng/mL)				
Males	17.1 +/- 2.1 (4)	2.8 +/- 0.2 (8)^{B}	1.9 +/- 0.1 (9)	1.6 +/- 0.1 (6)
Females	40.1 +/- 6.5 (2)	2.5 +/- 0.2 (8)	0.8 +/- 0.1 (4)	0.7 +/- 0.1 (5)
Glucose (mg/dL)				
Males	158.3 +/- 9.3 (4)	165.3 +/- 4.9 (10)	80.3 +/- 2.3 (7)	89.1 +/- 2.5 (8)
Females	282.6 +/- 32.3 (2)	123.1 +/- 4.7 (8)^{A}	87.3 +/- 3.9 (5)	91.9 +/- 2.0 (9)
Corticosterone (ng/mL)				
Males	238.5 +/- 17.9 (4)	134.9 +/- 40.2 (3)	54.0 +/- 4.2 (6)	63.4 +/- 2.7 (11)
Females	469.4 +/- 32.7 (2)	195.9 +/- 16.1 (8)^{B}	208.8 +/- 25.1 (5)	108.5 +/- 9.3 (9)
Estradiol (pg/mL)				
Females	3.9 +/- 0.3 (2)	6.8 +/- 0.8 (3)	ND	ND

$^{A}P< 0.005$ vs. knockout, $^{B}P< 0.05$ vs. knockout, NDNot Determined

Table 3.1 Body composition and neuroendocrine function in $ObR^{SynI}KO$ and $ObR^{Alb}KO$ mice. After sacrifice, the subset of significantly obese $ObR^{SynI}KO$ mice and the subset of $ObR^{Alb}KO$ mice with less than 30% ObR RNA as well as heterozygote littermates were exsanguinated and plasma was collected for the following assays. All plasma was collected from mice sacrificed at 1200 hrs under free feeding conditions. Average ages for $ObR^{SynI}KO$ and $ObR^{Alb}KO$ mice sampled here are 26 weeks and 23 weeks. All data represent the mean +/- SEM, with the sample size indicated in parentheses (^{A}, $p < 0.005$, ^{B}, $p < 0.05$ in an unpaired Student's t test).

$ObR^{SynI}KO$ males. Plasma corticosterone was elevated 1.8-fold in $ObR^{SynI}KO$ males and 2.4-fold in $ObR^{SynI}KO$ females ($p < 0.05$). The elevation in plasma glucose and the greater elevation in corticosterone specific to females could be due to the fact that the females analyzed had a lower level of ObR RNA and were more obese. Of note, an increased plasma corticosterone is unique to *ob/ob* and *db/db* mice, whereas other forms of rodent obesity are not generally associated with elevated corticosterone (Bray and York 1979). Mutations in leptin or its receptor are also associated with infertility, and leptin has been shown to influence the hypothalamic-pituitary-gonadal axis (Chehab et al. 1997). Consistent with this, obese female $ObR^{SynI}KO$ mice had 43% reductions in plasma estradiol. Male $ObR^{SynI}KO$ mice, however, did not have reduced levels of plasma testosterone. This sexual dimorphism is consistent with the fact that leptin deficiency has a more profound effect on female reproduction than male reproduction (Ewart-Toland et al. 1999). Finally, plasma thyroxine and triglycerides were not significantly different between mutant and control mice (data not shown).

Despite the marked obesity in this subset of $ObR^{SynI}KO$ mice, these animals still weighed less than mice with germline deletions of ObR (Figure 3.4a, $p < 0.05$ at all ages). These data suggest either that the knockout was incomplete even in the most obese animals or that leptin also acts at peripheral sites to reduce weight. To further assess the extent of the knockout in brain, levels of a number of hypothalamic neuropeptides were examined using Taqman assays (Figure 3.4b). Defective leptin signaling is associated with increased levels of hypothalamic AGRP, NPY, and MCH RNA and reduced levels of POMC and CART RNA (Stephens et al. 1995; Qu et al. 1996; Ollmann et al. 1997;

Shutter et al. 1997; Thornton et al. 1997; Kristensen et al. 1998). Comparisons of heterozygotes to obese $ObR^{SynI}KO$ mice showed that AGRP and NPY levels were increased, while POMC and CART levels showed a trend towards being reduced. While the reduction in POMC and CART RNA levels is not significant, the levels of these RNAs are only modestly decreased in *ob/ob* and *db/db* hypothalamus. MCH levels were indistinguishable between heterozygous mice, $ObR^{SynI}KO$ mice, and ObR null mice. The reported 80% increase in MCH RNA in *ob/ob* mice is also more modest than the reported increases in AGRP and NPY RNA (Qu et al. 1996). While the levels of AGRP and NPY were increased in the obese $ObR^{SynI}KO$ mice, they were less elevated than in null mice. This suggests that, despite the low levels of ObR RNA in these mice, some ObR expressing neurons remained.

Hepatocyte-specific ObR knockout

The data here indicate that leptin has direct effects on brain but leaves open the possibility that leptin also has direct effects on peripheral tissues. To assess a possible role of ObR in liver, a peripheral tissue that has been suggested to be a direct target of leptin action, mice with a hepatocyte-specific knockout of ObR were generated ($ObR^{Alb}KO$). These mice were generated using the same breeding strategy as described for $ObR^{SynI}KO$ mice (Figure 3.2a). The albumin promoter was used to direct hepatocyte-specific expression of Cre recombinase (Figure 3.5a) (Pinkert et al. 1987). Genomic PCR performed on multiple tissues from $ObR^{flox/+}$ albumin-Cre (+) mice confirmed that cre-mediated recombination was restricted to liver (Figure 3.5b). In contrast to $ObR^{SynI}KO$ mice, $ObR^{Alb}KO$ mice did not manifest an increased weight even in those animals where

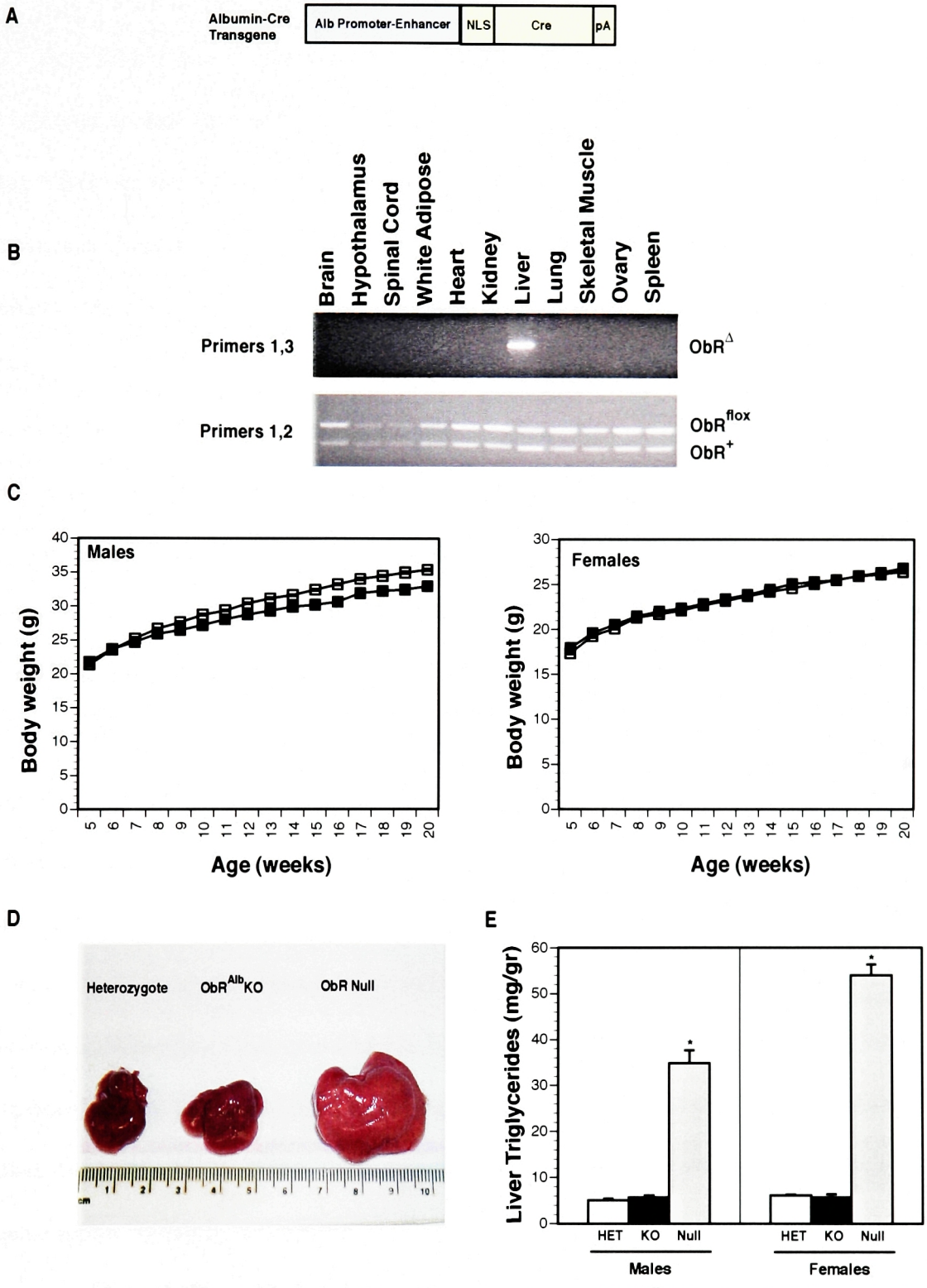


Figure 3.5 Normal body weight and liver phenotype in ObR^{Alb}KO mice. (A) Schematic of Albumin-Cre transgene. (B) Genomic DNA was prepared from several tissues from ObR$^{flox/+}$, Alb-Cre(+) mice and PCR amplified using primers flanking the first coding exon of ObR as described in Figure 5.2B. (C) Weight curves of male and female ObR^{Alb}KO mice (solid squares) and heterozygote littermates (open squares). Mice were weighed weekly from 5 weeks of age. Data represent the mean +/- SEM of 9 male and 11 female ObR^{Alb}KO mice and 23 male and 23 female heterozygote littermates. For both sexes, ObRAlbKO vs. heterozygotes, p values were not significant at all ages. (D) Photographs of freshly dissected livers from representative mice with the given genotypes. (E) Liver triglycerides (mg triglyceride / gram liver) were determined for ObR^{Alb}KO mice with < 30% of wild type ObR RNA and heterozygote controls. Data represent the mean +/- SEM of 6 male and female ObRAlbKO mice, 5 male and 5 female heterozygote controls, and 6 male and 3 female ObR null mice. For both sexes, $p < 0.05$ for ObRAlbKO and

the levels of ObR RNA in liver (as measured using the aforementioned Taqman assay) were reduced to less than 30% wild type levels (9/12 males, 11/16 females analyzed) (Figure 3.5c). As the liver is composed of a number of different cell types, and 80% of the mass is accounted for by hepatocytes, this likely represents a near complete deletion in hepatocytes (Weibel et al. 1969; Gumucio et al. 1996). Inspection of the weight distributions showed that no $ObR^{Alb}KO$ mice manifested an increased weight. Body composition was analyzed to determine whether hepatocyte-specific deletion of ObR had a subtle effect on body partitioning in $ObR^{Alb}KO$ mice. Water mass, lean mass, and fat mass were all unchanged in these animals (Table 3.1). In both sexes, plasma leptin levels were within the normal range as were insulin, glucose, and corticosterone. While *db/db* and $ObR^{Syn}KO$ mice have enlarged fatty livers characteristic of leptin deficient mice, the livers of $ObR^{Alb}KO$ mice were grossly normal (Figure 3.5d). Wet liver weight and liver weight as a percentage of body weight were also unchanged between $ObR^{Alb}KO$ mice and heterozygotes (data not shown). Quantitation of liver triglycerides showed no difference between $ObR^{Alb}KO$ mice and heterozygote controls, while levels in ObR null mice were significantly elevated relative to both of these groups ($p < 0.05$) (Figure 3.5e). These data indicate that ObR expressed in hepatocytes is not likely to play a significant role in body weight homeostasis or the hepatic steatosis associated with the *db* mutation, and that the liver abnormalities observed in *ob/ob* and *db/db* mice are secondary to defective leptin signaling in the brain.

In this study, two possible sites of leptin action were evaluated by analyzing the phenotype of mice with neuron and hepatocyte-specific knockouts of the leptin receptor. These data provide direct genetic evidence that leptin signals via direct effects on neurons. Separate data from studies of db^{Kls}/db^{Kls} mice indicate that the specific absence of the ObRb form of the receptor results in a phenotype indistinguishable from leptin deficiency, demonstrating that ObRb is absolutely required for the weight reducing effects of the hormone (Tartaglia et al. 1995; Chen et al. 1996b; Lee et al. 1996). ObRb is the only isoform that has all of the motifs necessary for signal transduction and is expressed at high levels in brain and a number of other tissues.

These findings suggest that leptin exerts its weight reducing and some, or perhaps all, of its neuroendocrine effects via interactions with the ObRb form of the receptor in specific classes of neurons in brain. This is consistent with the high potency of leptin administered i.c.v. and the anatomic distribution of ObRb (see below) (Campfield et al. 1995; Halaas et al. 1995). This conclusion is also consistent with data from experiments where an NSE-ObRb transgene was able to partially complement the phenotype of *db/db* mice (Kowalski et al. 2001). However, in this study ObRb was overexpressed 30-fold in all neurons, with some leakiness in peripheral tissues.

The phenotype of the obese $ObR^{Syn}KO$, while significant, did not reach that of ObR null mice. However, in these animals, hypothalamic AGRP and NPY levles, while increased, were still not as high as in null mice suggesting that there were still some residual ObR expressing cells. This leaves open the possibility that a truly complete brain-specific knockout (i.e. deletion of ObR on every neuron) could recapitulate the

obese phenotype of ObR null mice. The generation of such a knockout might require the simultaneous use of multiple different neuron-specific cre expressing lines.

In some studies, the expression of ObRb in brain was reported to be restricted to the hypothalamus, possibly implicating the hypothalamus as the principal target of leptin's weight reducing effects. In hypothalamus, ObRb is enriched in the arcuate, ventromedial, dorsomedial, and paraventricular hypothalamic nuclei, all of which are known, based on studies of animals with stereotactic lesions, to play a role in regulating food intake and body weight (Hetherington and Ranson 1942; Elmquist et al. 1997; Fei et al. 1997; Elmquist et al. 1998a). However, in other studies, ObRb expression has also been reported in other brain sites including the brainstem, thalamus, and cerebellum. The possibility that one or more of these extrahypothalamic brain sites play important roles in the regulation of body weight can be tested using region-specific or neuron-specific promoters to drive Cre expression in localized brain areas and specific cell types. In the hypothalamus, ObRb is expressed in functionally distinct classes of neurons, the best characterized being neurons that co-express either NPY or ∝MSH (Mercer et al. 1996a; Cheung et al. 1997). Both of these neuropeptides exert potent effects on food intake and body weight. Experiments to test the effects of ObR deletion in these two types of neurons may allow an analysis of the functional importance of these two neuropeptides as downstream effectors of leptin signaling and could establish the extent to which these two cell types by themselves can fully account for the neural response to leptin.

A number of studies have suggested that leptin also has direct effects on liver (Shimabukuro et al. 1997a; Kim et al. 2000). The data presented here indicate that hepatocyte-specific deletion does not have any observable effects on body weight or body

composition and does not result in any gross liver pathology. Furthermore $ObR^{Alb}KO$ mice have equivalent amounts of liver triglycerides as controls, indicating that the hepatic phenotype of *db/db* mice is secondary to defective leptin signaling in brain. However, hepatocyte-specific deletion may have less obvious effects on hepatic lipid and carbohydrate metabolism. In addition, these findings do not exclude the possibility that hepatocyte ObR by itself serves functions, such as modulating leptin turnover.

While these findings suggest that neurons are required for leptin's effects on body weight, they do not exclude the possibility that leptin also directly acts on other peripheral targets. In addition to regulating food intake, body weight, and the neuroendocrine axis, leptin can also modulate the immune system, bone turnover, angiogenesis, and lipid metabolism (Lord 1998; Sierra-Honigmann et al. 1998; Ducy et al. 2000; Atkinson et al. 2002; Minokoshi et al. 2002). Indeed, ObRb is expressed in a number of peripheral tissues and direct effects of leptin on T cells, macrophages, pancreatic ß-cells, skeletal muscle, heart, and other cell types have been reported (Shimabukuro et al. 1997a; Kim et al. 2000; O'Rourke et al. 2001; Atkinson et al. 2002; Minokoshi et al. 2002). Other forms of ObR (ObRa, c, d, e) may play a role in transport of leptin across the blood brain barrier and choroid plexus, sites that express high levels of ObR. The function of ObR at these and other sites can now be assessed by crossing $ObR^{flox/flox}$ mice to transgenic lines that express Cre in a tissue-specific fashion. Such studies should allow an assessment of the relative role of direct effects of leptin on these tissues vs. indirect effects via signaling in brain. In conclusion, these findings indicate that the brain is a direct target for the weight reducing and neuroendocrine effects of

leptin and that the liver pathology of *ob/ob* and *db/db* mice is secondary to defective leptin signaling in neurons.

Chapter 4: Leptin-specific Programs of Gene Expression in Liver

Introduction

The adipocyte-derived hormone leptin functions as the afferent signal in an endocrine loop regulating energy homeostasis. Mice with mutations in leptin (*ob/ob*) or its receptor (*db/db*) exhibit marked obesity, hyperphagia, and endocrine abnormalities (Zhang et al. 1994; Tartaglia et al. 1995; Chen et al. 1996b; Lee et al. 1996). When administered to wild-type and *ob/ob* mice, leptin causes significant reductions in food intake and body weight (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995). These findings have implicated leptin as a key regulator of energy balance (Friedman and Halaas 1998). Current evidence indicates that the brain is a critical site of leptin action, suggesting that leptin modulates a neuronal circuit that controls food intake and peripheral metabolism (Chapter 3)(Cohen et al. 2001). At present, however, the components of this neuronal circuit and the nature of the efferent signals from the central nervous system that regulate metabolism in response to leptin are not well characterized.

Leptin elicits a metabolic response that cannot be explained by its effects on food intake alone. When the food intake of an *ob/ob* mouse is restricted to the amount of food voluntarily consumed by a leptin-treated mouse (pair-fed), the pair-fed animal loses significantly less weight than a leptin treated counterpart (Levin et al. 1996). Animals treated with leptin also exhibit a number of physiological differences from food-restricted animals. While pair-feeding causes the breakdown of both lean and fat mass, leptin treatment selectively reduces fat mass (Halaas et al. 1995; Pelleymounter et al. 1995;

Halaas et al. 1997). In addition, leptin treatment does not produce an increase in circulating free fatty acids and ketone bodies typical of starvation (Chen et al. 1996a; Shimabukuro et al. 1997a). Leptin treatment also eliminates the compensatory drop in energy expenditure induced by starvation (Halaas et al. 1997). In aggregate, these findings suggest that leptin causes weight loss by enacting a novel metabolic program, distinct from food restriction on both a physiological and molecular basis.

The mechanism underlying leptin's unique metabolic effects is likely to involve modulation of gene expression in metabolically active organs such as liver, adipose tissue, and muscle. In order to more fully characterize this metabolic response, data is presented here on leptin-specific programs of gene expression in liver. First, we examined the effects of total leptin deficiency on gene expression, by using oligonucleotide microarrays to compare *ob/ob* to wild-type liver. Next, gene expression was analyzed in *ob/ob* liver over a time course of leptin replacement and pair-feeding to identify unique profiles of gene expression characteristic of each state. Finally, the transcriptional regulation of clusters of genes with leptin-specific patterns of expression was examined.

Results

Comparison of gene expression in *ob/ob* and wild-type liver by oligonucleotide microarrays

Initially, suppressive subtractive hybridization was used to compare gene expression in *ob/ob* and wild-type liver (Diatchenko et al. 1996). Although this method successfully identified a few clones with altered expression in *ob/ob* liver, high-density

microarrays permit a more comprehensive and high-throughput analysis of gene expression, allowing one to compare the RNA levels of thousands of genes simultaneously (Brown and Botstein 1999; Lockhart and Winzeler 2000). Using Affymetrix murine 6500 Gene Chips, we examined the expression of roughly 6500 genes in the livers of *ob/ob* and lean littermate control mice. Based on the GeneChip 3.1 Software, 177 genes were upregulated and 98 genes were downregulated in *ob/ob* liver.

Table 4.1 shows all genes with more than 3-fold altered expression in *ob/ob* liver. The gene most upregulated in *ob/ob* liver was apoliprotein A-IV (17.5-fold), which functions in HDL metabolism. Many of the other molecules overexpressed in *ob/ob* liver are involved in lipid and carbohydrate metabolism, which was expected since *ob/ob* mice demonstrate increased hepatic lipogenesis and gluconeogenesis (Yen et al. 1976; Kaplan and Leveille 1981; Yoon et al. 2001). The gene most downregulated in *ob/ob* liver was insulin-like growth factor binding protein-2 (27.7-fold), and studies on this gene are the subject of Chapter 7. Other molecules with decreased expression include 3 major urinary proteins, 2 proteasome components, and 2 DNA binding proteins (Stat1 and NF-κB). Interestingly, targeted knockout of aromatase, which is downregulated 3.2-fold, leads to obesity and other metabolic abnormalities (Murata et al. 2002).

To assess the reliability of the microarray data, a number of Northern blots were performed on differentially expressed genes (Figure 4.1). All genes found to be differentially expressed by the microarrays were confirmed as being altered by Northern blotting. In fact, Northern blot analysis suggested that the GeneChip 3.1 Software might underestimate the true fold change values. Based on this analysis, any gene called I or D (increased or decreased) and called P (present) in at least one of the samples by the

Upregulated in *ob/ob* liver

Fold Change	Avg Diff Change	Genbank Accession	Name	Function	Northern Verified
17.5	4414	M64250	apolipoprotein A-IV	Lipid Metabolism	√
11.8	1104	AA059763	tubulin, beta 2	Structural	
10.2	901	AA073296	ATP citrate lyase	Lipid Metabolism	√
9.7	1367	W13002	L14 lectin	Binding Protein	√
~8.1	202	X03690	Ig heavy chain constant region mu(b)	Immune	
6.9	323	W33415	ATP citrate lyase	Lipid Metabolism	√
~6.6	473	M26270	stearoyl-CoA desaturase-2	Lipid Metabolism	√
6.3	6919	AA139907	spot14	Lipid Metabolism	√
~6.1	433	M61737	adipocyte-specific FSP27	Unknown	
~4.9	202	X15842	c-rel	DNA Binding	
~4.6	246	U72881	RGS-r	Signal Transduction	
4.4	611	J02652	malic enzyme	Lipid Metabolism	
4.3	1069	W91509	ATP citrate lyase	Lipid Metabolism	√
4.2	2172	W17745	ATP citrate lyase	Lipid Metabolism	√
~4.2	169	X91824	small proline-rich protein 1A	Cell Membrane	
4.1	2635	X13135	fatty acid synthase	Lipid Metabolism	
3.9	622	X95280	G0S2-like protein	Unknown	
3.9	618	D10024	protein-tyrosine kinase substrate p36 / calpactin I heavy chain	Signal Transduction	
3.8	1572	D64160	retinoic acid early transcript 1, alpha	Membrane Protein	√
3.8	488	W07946	homologous to alpha-1-antiproteinase F precursor	Secreted	
3.8	257	AA048650	hydroxysteroid (17-beta) dehydrogenase 12	Steroid Metabolism	
~3.6	174	AA104086	homologous to zinc finger protein 97	DNA Binding	
~3.6	134	X59846	GAS6	Growth Arrest	
3.5	1089	M77003	glycerol-3-phosphate acyltransferase	Lipid Metabolism	
3.4	2307	Z22216	apolipoprotein CII	Lipid Metabolism	√
~3.4	163	AA114781	homologous to UMP-CMP kinase	Nucleotide Metabolism	
~3.4	127	AA008737	peroxin 5	Peroxisome Targeting	
3.3	1355	U00445	glucose-6-phosphatase	Carbohydrate Metabolism	√
3.3	537	J04696	glutathione S-transferase class mu	Detoxification	
3.2	393	AA030483	homologous to insulin induced protein 2	Unknown	
~3.2	149	AA097626	homologous to endogenous murine leukemia virus	Unknown	
~3.1	108	X04123	terminal deoxynucleotidyltransferase	DNA Repair	
~3.0	104	AA000410	ATP citrate lyase	Lipid Metabolism	√

Downregulated in *ob/ob* liver

Fold Change	Avg Diff Change	Genbank Accession	Name	Function	Northern Verified
-27.7	-3160	L05439	insulin-like growth factor binding protein 2	Binding Protein	√
~-8.4	-627	D28132	PACAP/VIP receptor (PACAPR-3)	Receptor	
-8.1	-2343	U22516	angiogenin precursor	Angiogenesis	√
-7.5	-1892	M16358	major urinary protein IV	Secreted	
~-5.7	-244	X70398	P311	Unknown	
~-5.7	-322	L11613	proteasome subunit Lmp2	Protein Turnover	
-4.6	-3055	L11333	carboxyesterase	Detoxification	√
~-4.6	-308	U15636	GTP binding protein GTP2	Signal Transduction	
-4.5	-9596	M16359	major urinary protein III	Secreted	
-4.4	-470	U44088	TDAG51	Signal Transduction	√
~-4.2	-168	U22519	angiogenin-related protein	Angiogenesis	
~-3.9	-248	AA120109	homologous to ERG2	Ion Channel	
~-3.7	-181	U06924	stat1	Signal Transduction	
-3.7	-1005	X67469	alpha-2 macroglobulin receptor	Receptor	
-3.4	-285	U22031	proteasome subunit Lmp7	Protein Turnover	
-3.3	-960	J00479	Ig gamma2a-b	Immune	
~-3.2	-185	D00659	aromatase p450	Steroid Metabolism	
~-3.1	-142	D49730	V1a arginine vasopression receptor	Receptor	
-3.1	-274	M57999	NF-kappa-B DNA binding subunit	DNA Binding	
-3.1	-581	U06431	polymeric immunoglobulin receptor	Immune	
-3.1	-367	M57891	complement component C2	Inflammatory	
-3	-3427	X03208	group 1 major urinary protein	Secreted	

Table 4.1 Genes with differential expression between *ob/ob* and wild-type liver. All genes with more than 3-fold differential expression are shown. The average difference change is a software-derived quantitative measure of the difference in expression. ~ indicates that expression in the other sample is undetectable. Function is based on literature searches.

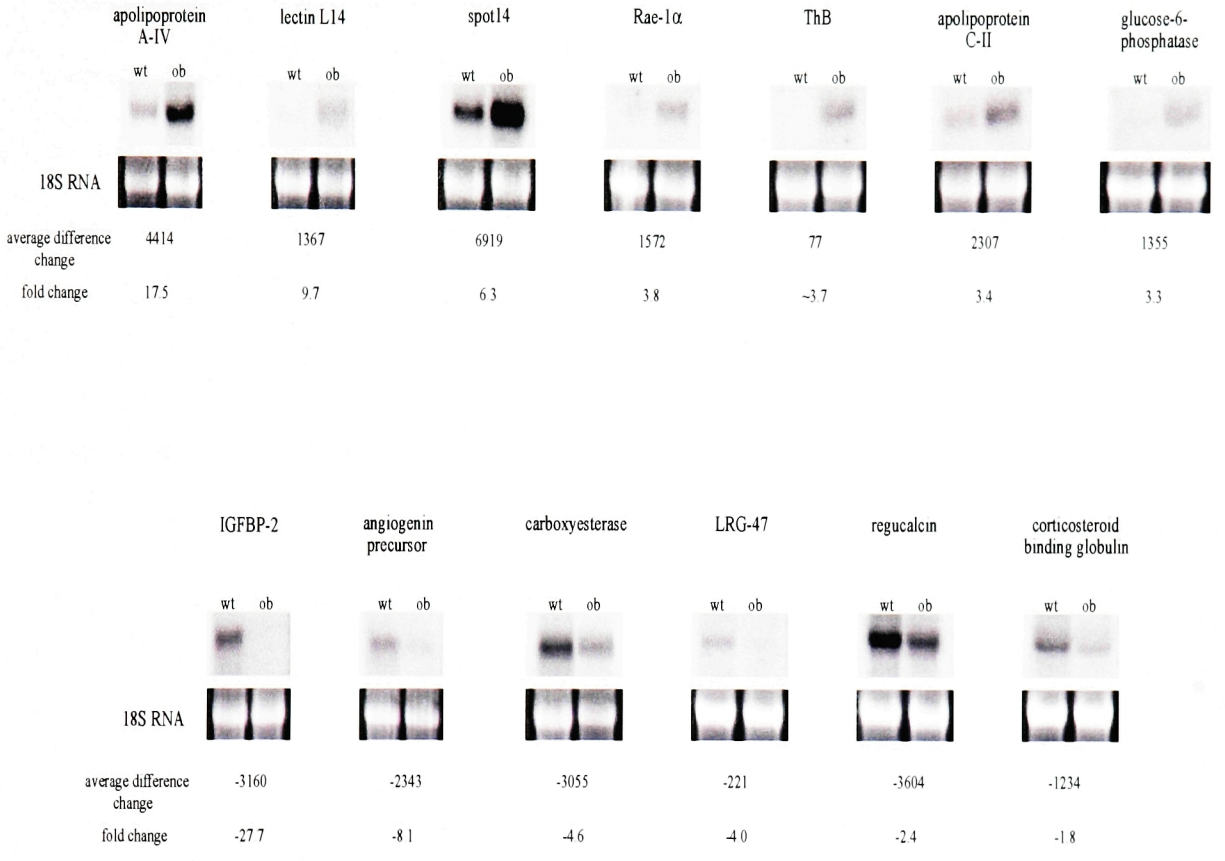


Figure 4.1 Northern blots of differentially expressed genes betweeen *ob/ob* and wild-type liver. Northern blots were performed to validate the microarray data using independently generated RNA. 10 g of total RNA was hybridized with a probe specific to the indicated genes. Equal loading was confirmed by ethidium bromide stained 18S RNA. The average difference change and fold change for each gene is indicated.

software, was considered to represent a reliable difference in RNA levels if the following criteria were also satisfied: (i) fold change $\geq$ 2.0 and average difference change $\geq$ 250, (ii) fold change $\geq$ 1.5 and average difference change $\geq$ 500, and (iii) fold change $\geq$ ~1.5 and average difference change $\geq$ 100 (the average difference change is a computational value of the magnitude of difference in expression). These thresholds were chosen conservatively to minimize the inclusion of false-positives in subsequent analysis. Based on these conditions, 79 genes were increased and 56 genes were decreased in *ob/ob* liver.

Leptin-specific patterns of gene expression

While binary comparisons, as described above, are a useful starting point for analysis, making biological conclusions from this data is extremely difficult. The altered gene expression noted in *ob/ob* mice may result from obesity, hyperphagia, leptin deficiency, or hormonal perturbations. Monitoring gene expression over a dynamic process, such as a time course, can be more useful for dissecting the molecular basis of a complex physiological pathway (Soukas et al. 2000; Soukas et al. 2001).

In order to better understand the basis for leptin's unique metabolic effects, we used microarrays to identify genes that are differentially regulated by leptin vs. pair-feeding in liver. To this end, *ob/ob* mice were followed over a time course of weight loss induced by either leptin administration or pair-feeding, as previously described (Soukas et al. 2000). Liver RNA was isolated from groups of *ob/ob* mice treated with subcutaneous leptin (infusion rate of 4.8 μg/24 hours) for 2, 4, and 12 days and freely fed *ob/ob* mice that received saline. This dose of leptin has been shown to produce plasma

levels of the hormone comparable to those in wild-type mice (Shimomura et al. 1999c). RNA was also isolated from *ob/ob* mice that were pair-fed to the leptin treated group for 2, 4, and 12 days of treatment. Adipose RNA was isolated from the same time course to analyze leptin-specific patterns of gene expression in this tissue (Soukas et al. 2000). The change in body weight and food intake of these groups of mice is shown in Figure 4.2 (a and b). As previously shown, leptin treated *ob/ob* mice lost significantly more weight than pair-fed mice indicating that leptin has metabolic actions that are not purely due to its effects on food intake (Levin et al. 1996).

Leptin replacement preferentially corrected the liver pathology in *ob/ob* mice. Leptin reduced hepatomegaly, significantly more so than pair-feeding ($p < 0.05$ leptin vs. pair-fed at days 2, 4, and 12) (Figure 4.2c). Leptin also caused a significant decrease in liver triglyceride levels, with far greater potency than pair-feeding (Figure 4.2d). This was further evident in histological sections showing a greater reduction in hepatic vacuolation following 12 days of leptin treatment than after an equivalent period of food restriction (Figure 4.2e).

RNA from the livers of these mice was hybridized to Affymetrix Murine 6500 GeneChips. Data were analyzed using a *K*-means clustering algorithm (Hartigan 1975; Kohonen 1997; Soukas et al. 2000). This computational method groups genes with similar patterns of expression over the time course experiment. For inclusion in the cluster analysis, a gene had to meet pre-established selection criteria, based on which 326 genes were included in the analysis (See Materials and Methods, Chapter 2) (Soukas et al. 2000; Soukas et al. 2001).

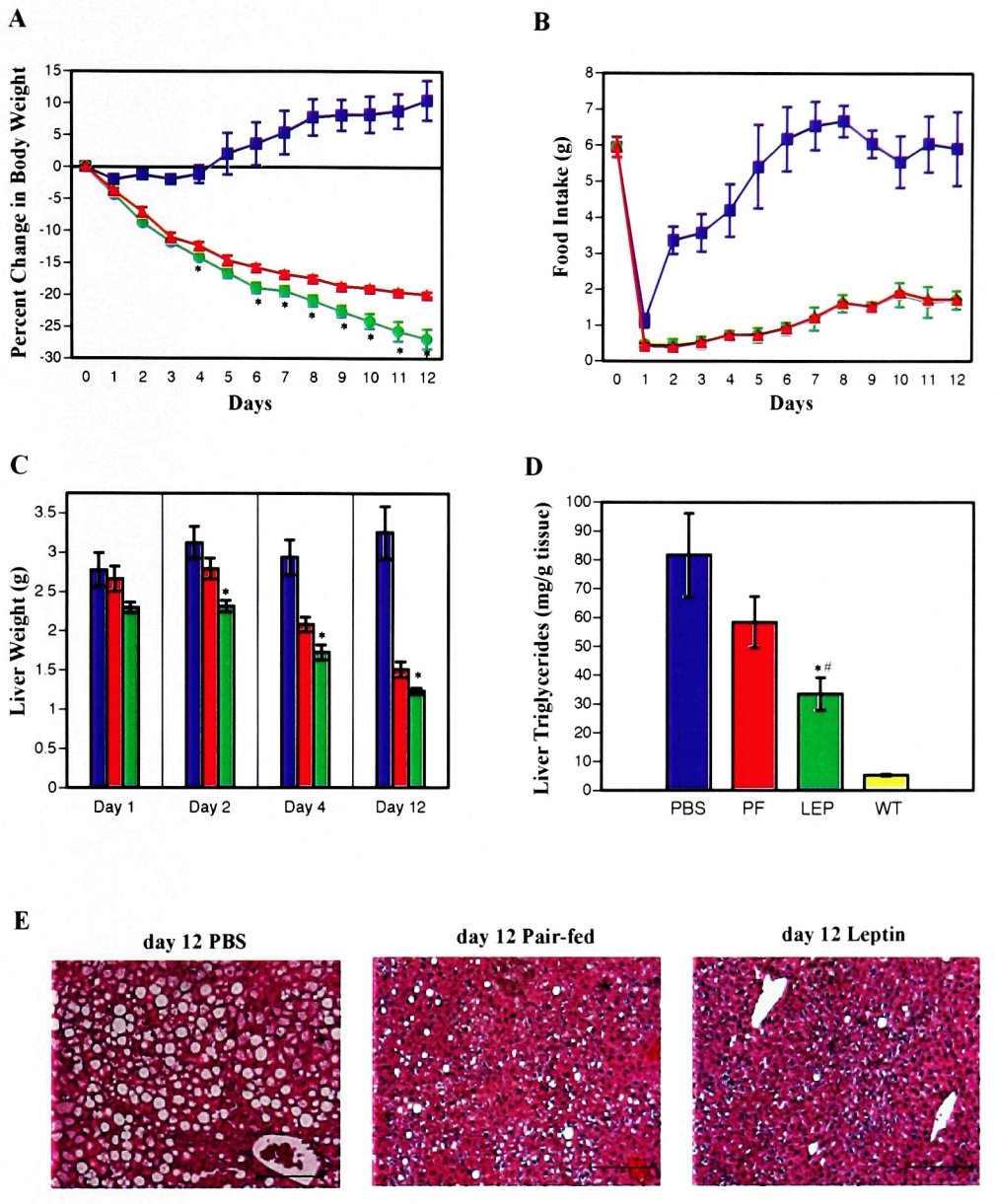


Figure 4.2 Leptin treatment produces novel metabolic effects. (A) Percent change in body weight and (B) daily food intake in *ob/ob* mice treated with leptin (green circles), PBS with pair-feeding (red triangles), and PBS with free-feeding (blue squares). Liver RNA was isolated from animals sacrificed at days 2, 4, and 12 of the time course. n = 4 per group, error bars denote SEM, *, $p < 0.05$ pair-fed vs. leptin. At all time points, except t0, both pair-fed and leptin values were significantly different from pbs values ($p < 0.01$). (C) Liver weight and (D) liver triglyceride content at day 12 of treatment and in untreated wild-type liver. For (C) and (D), PBS is in blue, pair-fed is in red, and leptin is in green; error bars denote SEM. For (C) n =4, *, $p < 0.05$ pair-fed vs. leptin, for (D) n =3, *, $p < 0.05$ leptin vs. pbs and $^{\#}$, $p = 0.07$ pair-fed vs. leptin. (E) Hematoxylin and eosin stained liver sections from representative mice sacrificed after 12 days of pbs, pair-feeding, or leptin. Images are 200x magnifications; scale bars denote 100 μm.

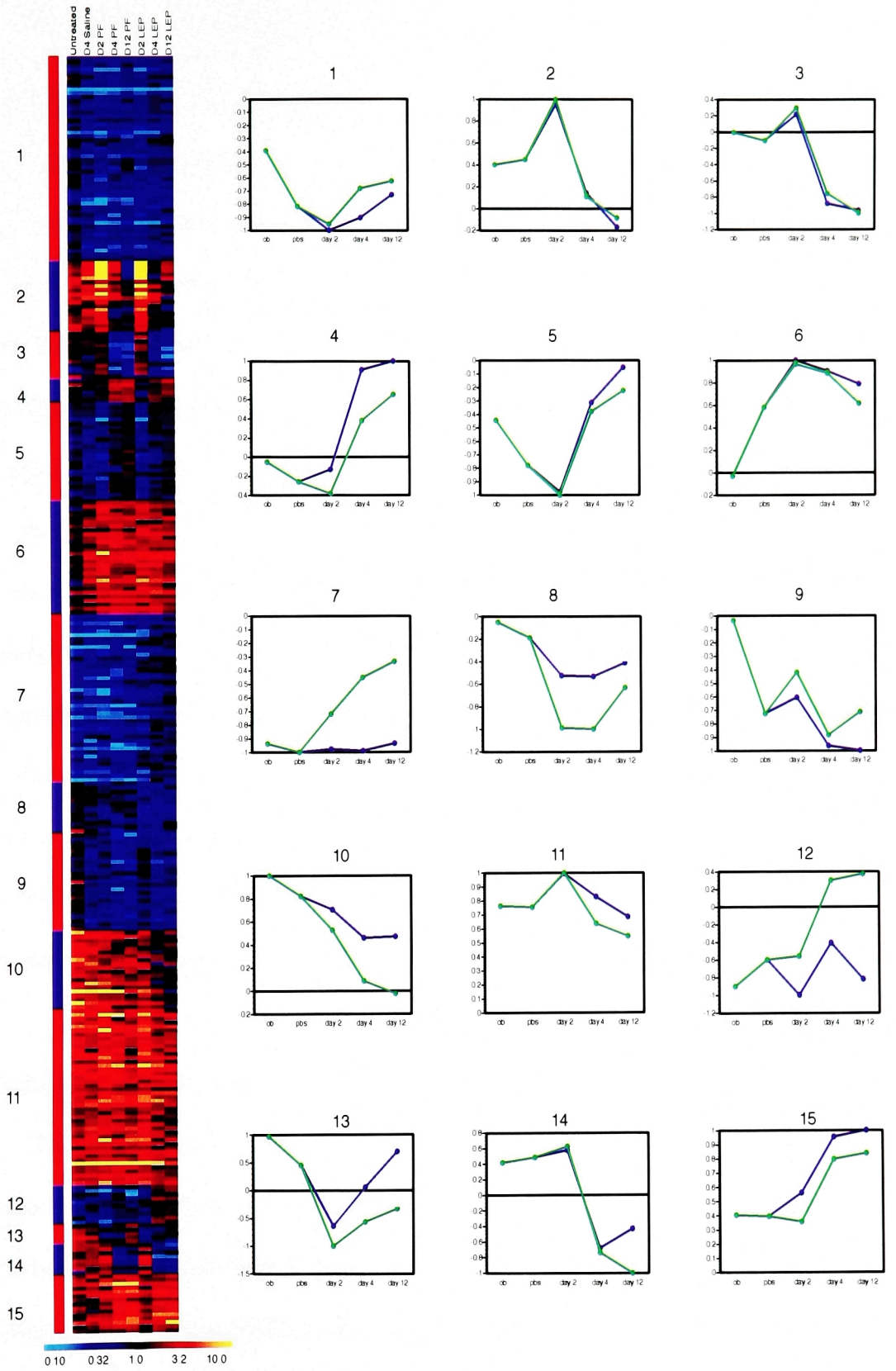


Figure 4.3 Leptin elicits a specific program of gene expression in *ob/ob* liver. K-means cluster analysis of genes from the time course. In the colorgram on the left, each row represents a gene and each column represents a group. The color denotes the expression level of each gene relative to wild type. The graphs on the right are another means of representing the data. In these curves, the expression profile of the average gene in each cluster is depicted relative to untreated lean littermate controls for which expression is given a value of zero. The expression levels in leptin treated and pair-fed mice are shown in green and blue respectively. 15 clusters of genes with distinct patterns of expression were identified, six of which correspond to genes that are specifically regulated by leptin relative to pair-feeding.

15 clusters of genes with distinct patterns of expression were identified, six of which corresponded to genes specifically regulated by leptin, but not pair-feeding (Figure 4.3). These clusters were likely to include genes that are either functional components or markers of leptin's metabolic effects. These six clusters were further subdivided into two classes: (a) genes that were overexpressed in *ob/ob* liver and specifically reduced by leptin (clusters 10, 11, 13, and 14) and (2) genes that were underexpressed in *ob/ob* liver and preferentially induced by leptin (clusters 7 and 12). In addition, several clusters of genes showed similar patterns of expression with both leptin treatment and pair-feeding, suggesting that these profiles represented a more general response to reduced food intake and weight loss or to experimental manipulation (Figure 4.3, clusters 2, 3, 5, 6, and 15). A complete list of the genes in all of the clusters can be found on the worldwide web (http://hal.rockefeller.edu/arrays/leptin/liver/). In aggregate, these data confirm that leptin elicits a novel program of gene expression in liver. As the genes in each of these clusters are specifically regulated by leptin (as compared to pair feeding), they could, in certain cases, mediate some of the effects of the hormone.

Transcriptional control of leptin-regulated clusters of genes

The leptin-regulated clusters, particularly those whose expression was specifically repressed by leptin (clusters 10, 11, 13, and 14), were enriched in genes involved in lipid and carbohydrate metabolism. Genes with roles in lipid metabolism included molecules involved in lipogenesis (SCD-1, SCD-2, malic enzyme, ATP citrate lyase, fatty acid synthase, spot14, and glycerophosphate dehydrogenase), lipid transport (CD36, PMP70),

and apolipoprotein metabolism (plasma phospholipid transfer protein, apolipoprotien A-IV, apolipoprotein C-II). Genes with functions in carbohydrate metabolism included the three rate-limiting enzymes of gluconeogenesis, glucose-6-phosphatase fructose bisphosphatase 1, and phosphoenolpyruvate carboxykinase 1 (PEPCK) (all members of cluster 10, Figure 4.3). The transcriptional regulation of lipogenesis and gluconeogenesis are both well characterized. Since leptin specifically repressed genes involved in these processes, we examined whether leptin regulated the transcription factors coordinating these metabolic pathways.

Sterol regulatory element–binding proteins (SREBPs) are a family of basic helix-loop-helix transcription factors that regulate the entire program of fatty acid and cholesterol biosynthesis (Horton et al. 2002). In their inactive form, SREBPs are bound to the endoplasmic reticulum membrane. In reponse to sterol depletion, SREBPs are proteolytically cleaved from the ER membrane and translocate to the nucleus, where they activate the transcription of genes containing sterol response elements (SREs)(Horton et al. 2002). Studies in transgenic mice have shown that SREBP-1a and –1c primarily activate transcription of fatty acid biosynthetic molecules, while SREBP-2 preferentially activates cholesterol biosynthetic genes (Shimano et al. 1996; Shimano et al. 1997; Horton et al. 1998). SREBP-1c RNA levels and nuclear protein were shown to be increased in diabetic, leptin deficient *ob/ob* and aP2-SREBP-1c transgenic mice (Shimomura et al. 1999a; Shimomura et al. 2000). Microarray analysis of SREBP expression, however, found only a small, unreliable increase in SREBP RNA levels in *ob/ob* mice and no substantial alterations upon either leptin administration or pair-feeding, a finding that was confirmed by Northern blot analysis (Figure 4.4a) and

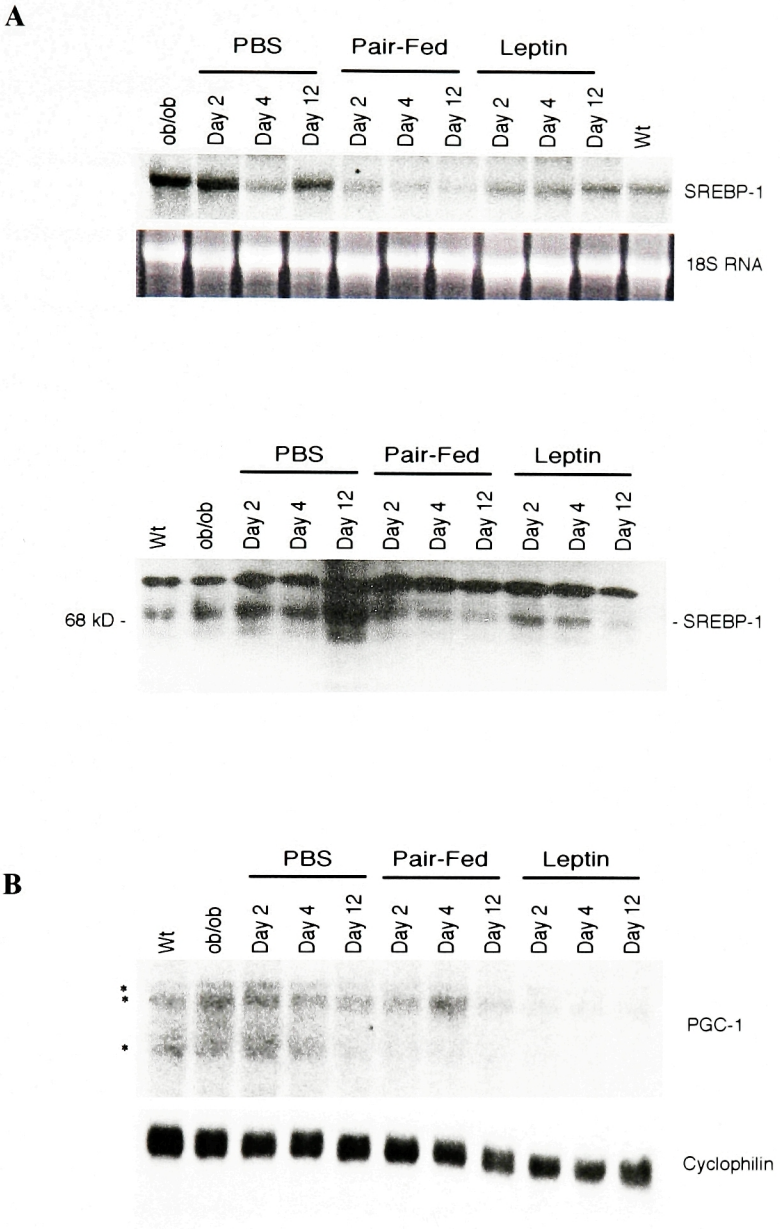


Figure 4.4 Transcriptional regulation of leptin-specific clusters of gene expression. (A) Northern blot analysis (top) of SREBP-1 RNA levels using a probe that hybridizes with both SREBP-1a and -1c. Ethidium bromide stained 18S RNA was used to confirm equal loading. Western blot analysis (bottom) of 68 kD nuclear SREBP protein. The upper band likely represents an intermidate of proteloytic cleavage. Nuclear protein was prepared as described in Chapter 2. (B) Northern blot analysis of PGC-1 RNA levels using a probe that hybridizes to all three splice variants, indicated by an * The blot was hybridized with a cyclophilin probe to confirm equal loading.

ribonuclease protection assays (data not shown). Furthermore, Western blotting detected no increase in nuclear SREBP-1 protein in *ob/ob* mice, and a minor, but equivalent repression following either leptin treatment or pair-feeding (Figure 4.4a).

Gluconeogenic gene expression is regulated by a number of factors, including the transcriptional cofactor PGC-1. This molecule was originally identified as a cold-induced transcriptional coactivator of PPARγ and thyroid hormone receptor in brown fat (Puigserver et al. 1998). Subsequent studies showed that the cAMP-regulated transcription factor CREB induced PGC-1, which in turn, coactivated HNF4α and induced gluconeogenic gene expression (Herzig et al. 2001; Yoon et al. 2001). In addition, in animal models of hepatic insulin resistance, including *ob/ob* mice, which fail to downregulate hepatic gluconeogenesis in response to elevated insulin, liver PGC-1 expression was elevated (Yoon et al. 2001). Northern blot analysis demonstrated leptin-specific repression of the RNA levels of all three PGC-1 splice variants, with no alteration observed in response to pair-feeding (Figure 4.4b). The pattern of PGC-1 expression in response to leptin treatment mirrored the expression profiles of glucose-6-phosphatase, fructose bisphosphatase 1, and phosphoenolpyruvate carboxykinase 1 (cluster 10, Figure 4.3). Thus, leptin-mediated repression of PGC-1 may be the mechanism by which leptin suppresses gluconeogenic gene expression.

Discussion

A number of reports have examined gene expression and its involvement in obesity and metabolic diseases. Differential display was done to identify alterations in

gene expression in *ob/ob* skeletal muscle, and global analysis of expression was performed in multiple tissues of *ob/ob* mice using the GeneCalling technology (Vicent et al. 1998; Renz et al. 2000). Oligonucleotide microarrays were used to characterize expression profiles in white adipose tissue in response to leptin treatment or over a gradient of increasing obesity and worsening diabetes (Nadler et al. 2000; Soukas et al. 2000). Microarrays were also used to study gene expression in *ob/ob* liver in response to leptin therapy, with similar findings to those described here (Ferrante et al. 2001; Liang and Tall 2001).

The liver is central to whole body metabolism and plays an important role in the determination to follow either an anabolic or catabolic fate. Lipogenic gene expression was elevated in *ob/ob* liver and was previously shown to be suppressed in *ob/ob* adipose tissue, an observation that was also noted in obese humans, and indicates that hepatic fatty acid biosynthesis may contribute substantially to the development and maintenance of obesity (Nadler et al. 2000; Soukas et al. 2000; Diraison et al. 2002). Livers of *ob/ob* mice are massively enlarged and steatotic, and contain elevated lipid and glycogen in keeping with an anabolic process. Weight loss and depletion of hepatic lipid require a shift to a catabolic program, which appears to be specifically modulated by leptin. Understanding the molecular basis for these programs could provide a marker for leptin's unique effects and could ultimately explain how body composition is determined.

The effects of leptin cannot be accounted for by a reduction in food intake alone, as leptin treated *ob/ob* mice lose significantly more weight than pair-fed controls (Levin et al. 1996). In addition, leptin replacement completely corrects the hyperinsulinemia in *ob/ob* mice, which is likely associated with enhanced hepatic insulin sensitivity, whereas

pair-feeding only reduces insulin levels by 50% (Levin et al. 1996). Thus, leptin-specific modulation of gene expression and the depletion of hepatic lipid may, in fact, be secondary to the correction of diabetes and neuroendocrine abnormalities of *ob/ob* mice. Leptin-deficient, lipodystrophic aP2-SREBP-1c transgenic mice also manifest hyperinsulinemia, diabetes, and hepatic steatosis, all of which can be corrected by leptin administration (Shimomura et al. 1999c). The alterations in gene expression and patterns of expression following leptin treatment of these mice demonstrate a striking similarity to those of *ob/ob* mice (E. Asilmaz, unpublished results). These findings suggest that leptin-deficiency accounts for a significant component of the abnormalities in both of these animal models and that the leptin-specific programs of gene expression observed in *ob/ob* and aP2-SREBP-1c transgenic mice may be secondary to the correction of hyperinsulinemia and other endocrine defects.

To study the effects of leptin, independent of its correction of the obese phenotype, gene expression was also analyzed in wild-type liver at several time points of leptin administration and pair-feeding. In wild-type mice, neither leptin treatment nor pair-feeding significantly affect insulin levels or other endocrine parameters (Levin et al. 1996). Only a small number of genes were specifically modulated by leptin in wild-type liver, further indicating that the leptin-specific transcriptional signature in *ob/ob* mice might be largely secondary to correction of the obese condition (data not shown). These genes are more likely to be specifically modulated by quantitative changes in circulating leptin levels.

Data presented earlier in this thesis (Chapter 3) suggested that a significant proportion of leptin's effects on hepatic gene expression were the result of central action.

In addition, i.c.v. leptin enacted a markedly similar program of gene expression to peripherally administered leptin in lipodystrophic mice (E. Asilmaz, unpublished results). The efferent pathways responsible for leptin's effects on gene expression and metabolism are not known, but are likely to involve the combined modulation of the autonomic nervous system and the hypothalamic-pituitary axis. Central administration of NPY stimulates peripheral lipogenic gene expression (Billington et al. 1991; Zarjevski et al. 1993). Leptin decreases NPY levels in the hypothalamus, suggesting that the modulation of a neuronal circuit downstream of NPY may be responsible for leptin's effects on lipogenic gene expression (Stephens et al. 1995). Genetically modified pseudorabies viruses have been used to trace the leptin circuitry within the brain (DeFalco et al. 2001). Similar studies are in progress to identify the neuronal sites from which efferents project to peripheral metabolic organs such as adipose tissue and liver.

Understanding the transcriptional mechanisms underlying leptin-specific patterns of gene expression may help identify the efferent signal(s) underlying leptin's peripheral effects. Based on the membership of leptin-regulated gene clusters, we examined whether leptin regulated the levels of known, candidate transcription factors. While previous reports indicated that levels of SREBP-1c RNA and nuclear protein were elevated in *ob/ob* mice, experiments performed here did not detect any alterations (Shimomura et al. 1999a; Shimomura et al. 2000). SREBP expression and cleavage are stimulated by insulin, and the discrepancy between our results and those previously published may reflect differences in insulin levels (Foretz et al. 1999; Shimomura et al. 1999a; Shimomura et al. 1999b). Specifically, the previous studies used older mice in the middle of the dark cycle, when insulin levels should be at their peak, whereas we studied

younger mice in the middle of the light cycle, when insulin levels are likely to be at their nadir (Shimomura et al. 1999a; Shimomura et al. 2000). Leptin was found to regulate SREBP RNA levels and cleavage in adipose tissue (Soukas et al. 2000). However, in adipose tissue a far more comprehensive list of SREBP-target genes than identified here were found to co-cluster, perhaps indicating that the lipogenic genes regulated by leptin in the liver could be targets of another transcription factor. We did find that leptin specifically downregulated the RNA levels of PGC-1 a transcriptional cofactor, that regulates gluconeogenic gene expression (Herzig et al. 2001; Yoon et al. 2001). As *ob/ob* mice are diabetic and have elevated hepatic gluconeogenesis, leptin-specific modulation of PGC-1 might contribute to the correction of diabetes associated with leptin treatment. Further studies are required to determine the role of this molecule in the transcriptional response to leptin. In addition to these molecules, leptin may also regulate the levels and/or activity of PPARα, PPARγ, ChREBP, HNFs, LXRs, or yet to be identified transcription factors (Bocher et al. 2002; Edwards et al. 2002; Schrem et al. 2002; Uyeda et al. 2002).

In aggregate, these data demonstrate the induction of a novel, distinct transcriptional signature characteristic of the unique metabolic program of weight loss induced by leptin. These data distinguish leptin treatment from food restriction at a molecular level, and may ultimately provide new insight into the nature of the leptin-stimulated efferent signals from the hypothalamus. A subset of these leptin-regulated genes may play a functional role in the obese phenotype and the unique metabolic actions of leptin.

Chapter 5: Role for Stearoyl-CoA Desaturase-1 in Leptin-Mediated Weight Loss

Introduction

Leptin regulates energy balance, metabolism, and the neuroendocrine response to altered nutrition (Zhang et al. 1994; Friedman 2000a). The metabolic program that leptin elicits is not explained by its effects on food intake alone (Levin et al. 1996; Kamohara et al. 1997). Leptin replacement in leptin-deficient (*ob/ob*) mice and humans leads to the depletion of lipid in adipose tissue, liver, and other tissues (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995; Farooqi et al. 1999). Leptin treatment also improves insulin sensitivity and reduces fat content in lipodystrophic mice and humans (Shimomura et al. 1999c; Oral et al. 2002).

To elucidate the mechanism whereby leptin reduces hepatic lipid content, we used microarrays to identify genes that were differentially regulated by leptin vs. pair-feeding in liver. Leptin treated *ob/ob* mice lose significantly more weight than pair-fed mice, indicating that leptin stimulates energy expenditure (Figure 4.2). Liver RNA from these animals was hybridized to microarrays and the data were analyzed using a *K*-means clustering algorithm (Soukas et al. 2000). 15 clusters of genes with distinct patterns of expression were identified, six of which correspond to genes specifically regulated by leptin, but not pair- feeding (Figure 4.3).

We used a more directed computational approach to identify genes that are particularly repressed during leptin-mediated weight loss. Leptin was found to

specifically repress RNA levels and enzymatic activity of hepatic stearoyl-CoA desaturase-1 (SCD-1), which catalyzes the biosynthesis of monounsaturated fatty acids. Mice lacking SCD-1 were lean and hypermetabolic. *ob/ob* mice with mutations in SCD-1 were significantly less obese than *ob/ob* controls and had markedly increased energy expenditure. *ob/ob* mice with mutations in SCD-1 had histologically normal livers with significantly reduced triglyceride storage and VLDL production. These findings suggest that down-regulation of SCD-1 is an important component of leptin's metabolic actions.

Results

Identifying and ranking leptin-repressed genes.

In order to prioritize leptin-regulated genes for detailed functional analysis, we developed an algorithm to identify and rank genes that are specifically repressed by leptin. This algorithm ranked genes based on the extent to which their expression was (1) increased in *ob/ob* liver compared to wild type (2) repressed by leptin treatment, and (3) different between leptin treatment and pair-feeding. Such genes are candidates as contributing to the obese phenotype. Specifically, we selected genes whose expression was increased in *ob/ob* relative to wild-type and corrected by leptin administration. Genes with the lowest scores are those most strongly leptin-regulated. The specific characteristics of this algorithm are described in more detail in Chapter 2. The results of this analysis are shown in Table 5.1. The gene encoding SCD-1 ranked the highest in this analysis.

Leptin-repressed gene expression in *ob/ob* liver

Accesion Number	Name	Function	Score
M21285	stearoyl-CoA desaturase-1	Lipid Metabolism	2.00
W29265	glutathione S-transferase, alpha 1	Detoxification	4.00
AA059763	tubulin, beta	Structural	5.50
J04696	glutathione S-transferase class mu	Detoxification	6.00
U00445	glucose-6-phosphatase	Carbohydrate Metabolism	7.75
J02652	malate NADP oxidoreductase	Lipid Metabolism	9.00
AA030483	est, similar to human insulin induced protein 2	Unknown	9.00
AA139907	spot14	Lipid Metabolism	10.00
W17745	ATP citrate lyase	Lipid Metabolism	14.50
D10024	calpactin I heavy chain	Signal Transduction	14.75
AA108647	fructose bisphosphatase 1	Carbohydrate Metabolism	14.00
M16465	calpactin I light chain	Signal Transduction	15.00
X62940	TSC-22	Unknown	16.00
W13002	lectin, galactose binding, soluble 1	Binding Protein	14.50
M26270	stearoyl-CoA desaturase-2	Lipid Metabolism	17.00
W83337	glutathione peroxidase 4	Detoxification	18.50
X13135	fatty acid synthase	Lipid Metabolism	20.00
AA028398	tubulin, beta	Structural	20.00
AA108166	fructose bisphosphatase 1	Carbohydrate Metabolism	20.00
M64250	apolipoprotein A-IV	Lipid Metabolism	20.50
W33721	enolase 1, alpha	Carbohydrate Metabolism	21.50
W13646	ubiquitin C	Protein Turnover	18.50
X61940	protein tyrosine phosphatase, non-receptor type 16	Signal Transduction	19.50
L28836	peroxisome membrane protein (PMP70)	Lipid Metabolism	23.00
D64160	retinoic acid early transcript 1, alpha	Membrane Protein	23.00
Z22216	apolipoprotein CII	Lipid Metabolism	24.50
W15891	ketohexokinase	Carbohydrate Metabolism	23.00
U37226	plasma phospholipid transfer protein	Lipid Metabolism	25.50
U29396	annexin V	Signal Transduction	25.00
D12618	nucleosome assembly protein-1	Chromatin Structure	23.50
M61737	adipocyte-specific FSP27	Unknown	28.00
M13366	glycerophosphate dehydrogenase	Lipid Metabolism	29.00
L23108	CD36	Lipid Metabolism	31.50
AA063800	phosphoenolpyruvate carboxykinase 1	Carbohydrate Metabolism	33.50
W62918	Epstein-Barr virus induced gene 3	Unknown	34.50
L09104	glucose phosphate isomerase	Carbohydrate Metabolism	37.00

Table 5.1 Leptin-repressed gene expression in *ob/ob* liver. In order to prioritize leptin-regulated genes for detailed functional analysis, an algorithm was developed to identify and rank genes that are specifically repressed by leptin. This algorithm ranked genes based on the extent to which their expression was (1) increased in *ob/ob* liver compared to wild type (2) repressed by leptin treatment, and (3) different between leptin treatment and pair-feeding. Such genes are candidates as contributing to the obese phenotype. Genes were selected whose expression was increased in *ob/ob* relative to wild-type and corrected by leptin administration. Genes with the lowest scores are most strongly leptin-repressed.

Leptin-specific regulation of SCD-1

SCD-1 is a microsomal enzyme that is required for the biosynthesis of the monounsaturated fats palmitoleate and oleate from saturated fatty acids (Ntambi et al. 1988; Ntambi 1999). SCD-1 RNA levels were highly elevated in untreated *ob/ob* liver (Figure 5.1a). SCD-1 RNA levels in leptin treated *ob/ob* mice were normalized at 2 days and by 4 days fell to levels below that of lean controls, a result consistent with previous studies (Ferrante et al. 2001; Liang and Tall 2001). Pair-fed mice showed a smaller and delayed decrease in SCD-1 gene expression.

SCD enzymatic activity was elevated 7.2-fold in livers of untreated *ob/ob* mice relative to wild type ($p < 0.0005$) and remained significantly elevated in saline-treated, free-fed *ob/ob* controls (Figure 5.1b). Leptin treatment normalized SCD enzymatic activity, while pair-feeding reduced enzymatic activity to a lesser extent ($p < 0.005$ leptin vs. free fed, $p < 0.02$ leptin vs. pair-fed at each time point). Levels of hepatic monounsaturated 16:1 and 18:1 fatty acids, the products of SCD-1, were elevated in *ob/ob* mice and normalized by 12 days of leptin treatment, but not by pair-feeding (Table 5.2). Leptin also preferentially normalized desaturation indices (ratio of 16:1/16:0 and 18:1/18:0 levels), which are an indicator of SCD enzymatic activity.

Reduced fat mass in mice lacking SCD-1

To investigate whether SCD-1 might mediate some of leptin's metabolic effects, we studied *asebia* mice (ab^J/ab^J), which carry mutations in SCD-1 (Gates and Karasek 1965). These mice have a spontaneous deletion of the first four exons of the SCD-1 gene

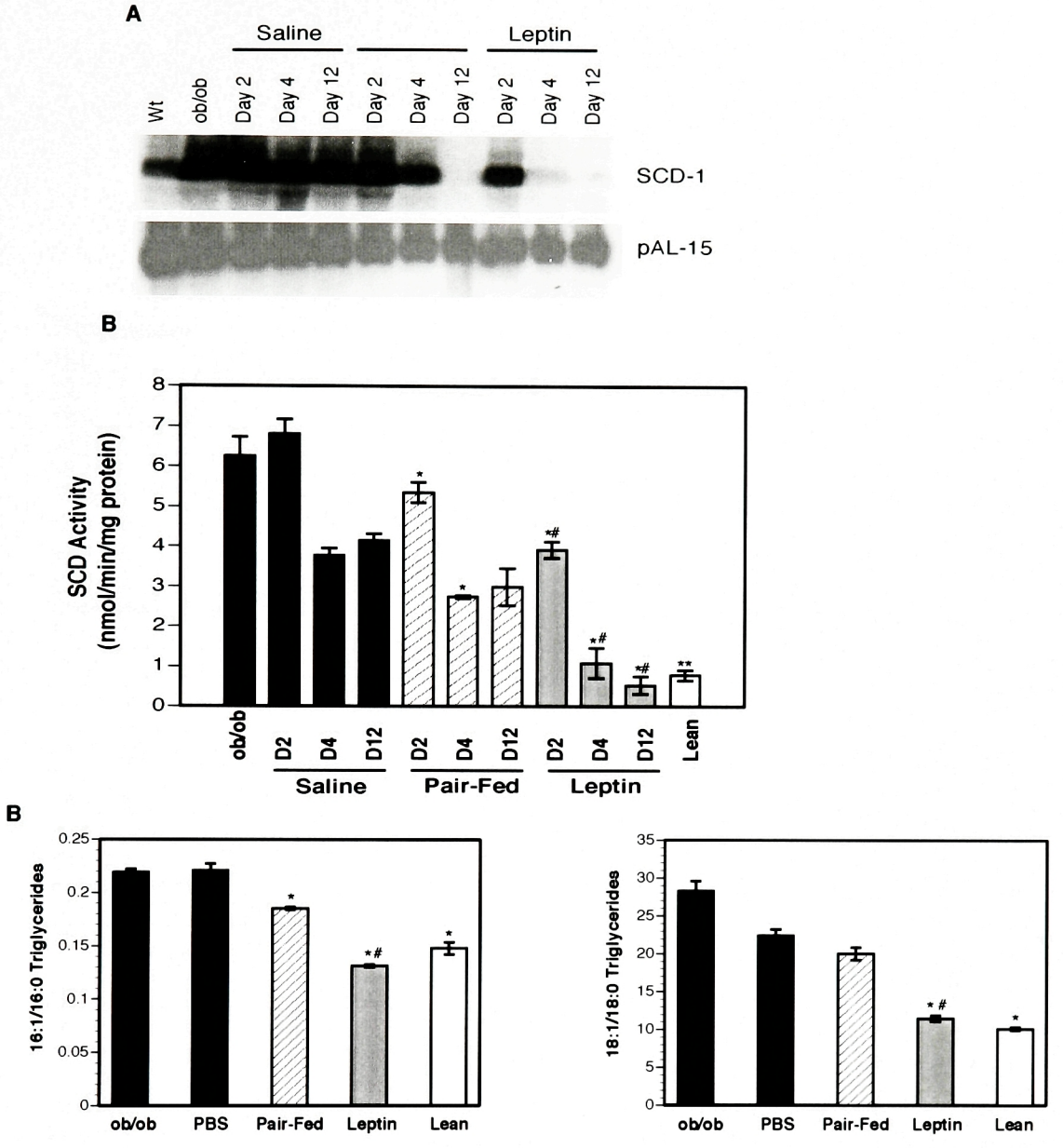


Figure 5.1 Leptin-specific down-regulation of SCD-1 RNA levels and enzymatic activity. (A) Northern blot of liver RNA samples from an independent time course experiment probed with radioactively labeled cDNA probes specific for SCD-1 and the small mitochondrial RNA pAL-15 (loading control). (B) SCD enzymatic activity assays measure the conversion of [1-^{14}C] stearoyl-CoA to [1-^{14}C] oleate, as described in Chapter 2. Activity is expressed as nmol min^{-1} mg^{-1} protein. Error bars indicate the SEM, n =3 for each group. *, $p < 0.05$ vs. saline treated, $^{\#}$, $p < 0.02$ vs. pair fed, ** $p < 0.0005$ untreated lean vs. untreated ob/ob. (C) Ratios of 16:1/16:0 and 18:1/18:0 triglycerides, as an indicator of SCD enzymatic activity. Values were determined from lipid composition analysis (Table 5.2). Error bars indicate the SEM, n =3 for each group. *, $p < 0.05$ vs. saline treated, $^{\#}$, $p < 0.05$ vs. pair fed.

Liver fatty acid composition

	Lean	ob/ob	Saline	Pair-Fed	Leptin
Triglycerides					
16:0	24.3	24.3	24.7	21.7*	21.8*
16:1n-7	3.6	5.3*	5.5	4.0*	2.9*#
18:0	3.2	1.8*	2.0	1.8*	2.6#
18:1n-9	32.0	49.2*	45.2	35.9*	29.4*#
18:1n-7	3.0	5.5*	4.9	3.8*	2.9*#
18:2n-6	20.3	6.0	9.1	20.4*	25.4*#
18:3 n-3	1.2	0.5*	0.9	1.6*	1.5*
20:4 n-6	1.0	0.1*	0.3	0.6*	1.2*#
22:6 n-3	3.6	0.3*	0.8	3.0*	5.5*#
Cholesterol Esters					
16:0	43.4	20.8*	25.0	23.0	31.2*#
16:1n-7	4.9	8.1*	7.0	9.4	5.8
18:0	8.2	4.0*	3.3	3.1	5.0#
18:1n-9	22.0	46.9*	40.6	36.8	30.3*
18:1n-7	1.7	5.3*	4.5	3.7	2.6*
18:2n-6	8.7	3.2*	6.6	11.5	11.8
18:3 n-3	0	0	0	1.4	1.0
20:4 n-6	0	0	0.3	0	1.3*#
22:6 n-3	1.2	0.5	0.9	1.2	2.3#
Phospholipids					
16:0	20.7	19.9	19.9	22.8*	24.2*#
16:1n-7	0.7	1.0	1.0	0.9	0.7*#
18:0	18.6	20.6*	21.3	17.9*	17.8*
18:1n-9	7.6	10.3*	8.6	6.5	6.0
18:1n-7	2.1	3.7*	2.9	2.2	1.6*#
18:2n-6	16.6	12.0*	13.3	17.0*	14.4#
18:3 n-3	0.4	0*	0.2	0.3	0.1
20:4 n-6	12.6	10.6*	10.3	13.4*	14.1*#
22:6 n-3	11.7	10.5*	12.3	12.5	14.9*#

Table 5.2 Liver fatty acid composition. Liver lipid composition was determined in untreated lean and *ob/ob* liver and following 12 days of pbs, pair-feeding, or leptin. Total lipids were extracted from liver with Folch solvent (chloroform/methanol 2:1). At the start of the liver extraction, internal standards of C17:0 triglyceride, cholesteryl ester, and phosphatidyl choline were added to quantitate each lipid class. The lipid classes in the liver extract were separated by thin layer chromatography using silica gel G and a developing solvent of hexane/diethyl ether/acetic acid (60:40:1). The fatty acids in each lipid class were methylated with fresh 5% methanolic HCl and analyzed with a gas chromatograph. Numbers in the table are averaged from 3 mice per group. For clarity, SEM is not shown, but all error values were within acceptable limits. *, $p < 0.05$ lean vs. ob/ob or $p < 0.05$ pair-fed or leptin vs. saline, #, $p < 0.05$ leptin vs. pair-fed.

and express no SCD-1 RNA or protein, though expression of SCD-2 is not altered (Zheng et al. 1999). SCD-1 is highly expressed in the sebaceous glands of the skin and the harderian and meibomian glands of the eye. Consequently, ab^J/ab^J mice have cutaneous and ocular defects. The phenotype of these mice is identical to that of mice with an induced mutation in SCD-1 (Miyazaki et al. 2001).

The weight of male ab^J/ab^J mice was indistinguishable from that of littermates and female ab^J/ab^J mice weighed significantly more than littermates (Figure 5.2a, $p < 0.005$ at all ages after 6 weeks). However, ab^J/ab^J mice had significantly reduced fat mass relative to controls (from 12.7% to 8.3% in females, $p < 0.005$; from 13.5% to 6.9% in males, $p < 0.0005$, Figure 5.2b) and plasma leptin levels (females: 2.3±0.4 ng/ml vs. 5.0±0.9 ng/ml, males: 2.7±0.5 ng/ml vs. 7.8±1.4 ng/ml, $p < 0.01$ for both sexes).

To explore the effects of SCD-1 deficiency on the *ob/ob* phenotype, we intercrossed *ob/+* and $ab^J/+$ or ab^J/ab^J mice. ab^J/ab^J; *ob/ob* mice showed a dramatic reduction in body weight at all ages compared to *ob/ob* littermate controls (Figure 5.2a, $p < 0.0001$ from 5 weeks of age). At 16 weeks of age, weight was reduced by 29% in females ($p < 10^{-6}$) and 34% in males ($p < 10^{-4}$). A photograph of a representative *ob/ob* and an ab^J/ab^J; *ob/ob* mouse is shown in Figure 5.2c. Fat mass in 16 week-old double mutant females was 32.1% vs. 51.0% in *ob/ob* ($p < 10^{-4}$) and 28.1% in double mutant males vs. 49.9% in *ob/ob* ($p < 10^{-5}$) (Figure 5.2b). ab^J/ab^J; *ob/ob* mice of both sexes also showed a significant increase in percent lean mass relative to *ob/ob* littermates (from 15.3% to 18.5% in females, $p < 0.005$; from 15.3% to 20.1% in males, $p < 0.05$) indicating that double mutants do not suffer from a generalized growth defect.

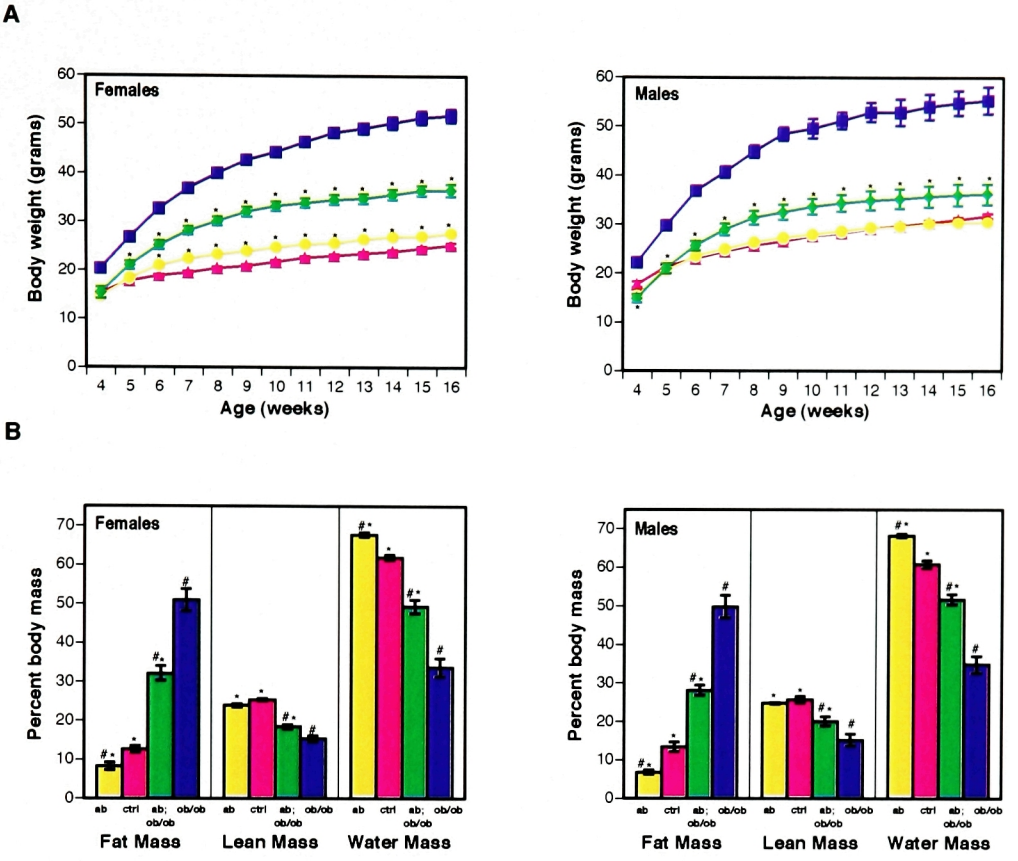


Figure 5.2 Attenuation of the obese phenotype in mice with a mutation in SCD-1 (*ab^J/ab^J*). Weight curves of *ob/ob* (blue squares), *ab^J/ab^J; ob/ob* (green diamonds), *ab^J/ab^J* (gold circles), and control (magenta triangles) mice. Error bars indicate the SEM, $n \geq 9$ for each group. *, $p < 0.01$ *ab^J/ab^J* vs. control or $p < 0.0001$ *ab^J/ab^J; ob/ob* vs. *ob/ob*. (B) Carcass analysis of female and male *ob/ob* (blue), *ab^J/ab^J; ob/ob* (green), *ab^J/ab^J* (gold), and control (magenta) mice was performed as in Chapter 2. Error bars indicate the SEM, $n \geq 8$ for each group. *, $p < 0.005$ vs. lean control, $^{\#}$, $p < 0.05$ vs. *ob/ob*. (C) Photograph of a representative *ob/ob* and *ab^J/ab^J; ob/ob* mouse.

Mechanism for reduced adiposity in mice lacking SCD-1

To analyze energy balance, we measured food intake and energy expenditure in *ob/ob* and lean littermates with and without homozygous SCD-1 mutations. *ob/ob* mice were hyperphagic compared to lean controls (Figure 5.3a,b). Despite being significantly leaner, ab^J/ab^J; *ob/ob* mice consumed 35% more food than *ob/ob* littermates (9.0±0.9 g/day vs. 6.6±0.4 g/day, $p < 0.05$) (Figure 5.3a,b). ab^J/ab^J mice consumed 53% more than lean controls (6.3±0.2 g/day vs. 4.2±0.2 g/day, $p < 10^{-6}$) with an average food intake indistinguishable from that of *ob/ob* mice (Figure 5.3a,b). Relative to *ob/ob* controls, ab^J/ab^J; *ob/ob* mice showed increases of 96% (female) and 56% (male) in total VO2 and increases of 174% (female) and 71% (male) in resting VO2 (Figure 5.3c,d, $p < 0.02$). Total and resting oxygen consumption in female double mutants were indistinguishable from those of lean controls and in male double mutants they were even greater than those of lean controls ($p < 0.05$ for resting VO2). Lean ab^J/ab^J mice expended more energy with increases of 30% and 46% in total oxygen consumption and increases of 43% and 62% in resting oxygen consumption for females and males respectively (Figure 5.3c,d, $p < 0.02$). Double mutant ab^J/ab^J; *ob/ob* mice had increased plasma levels of ketone bodies (β-hydroxybutyrate) relative to *ob/ob* littermates, suggesting increased fatty acid oxidation (females: 6.6±1.2 mg/dl vs. 3.4±0.6 mg/dl, $p < 0.05$; males: 4.5±1.4 mg/dl vs. 3.5±0.7 mg/dl, NS).

ob/ob mice have massively enlarged livers that are engorged with lipid. Gross inspection revealed that the hepatomegaly and the steatosis of *ob/ob* mice were normalized in ab^J/ab^J; *ob/ob* mice. Histological sections of *ob/ob* liver showed large

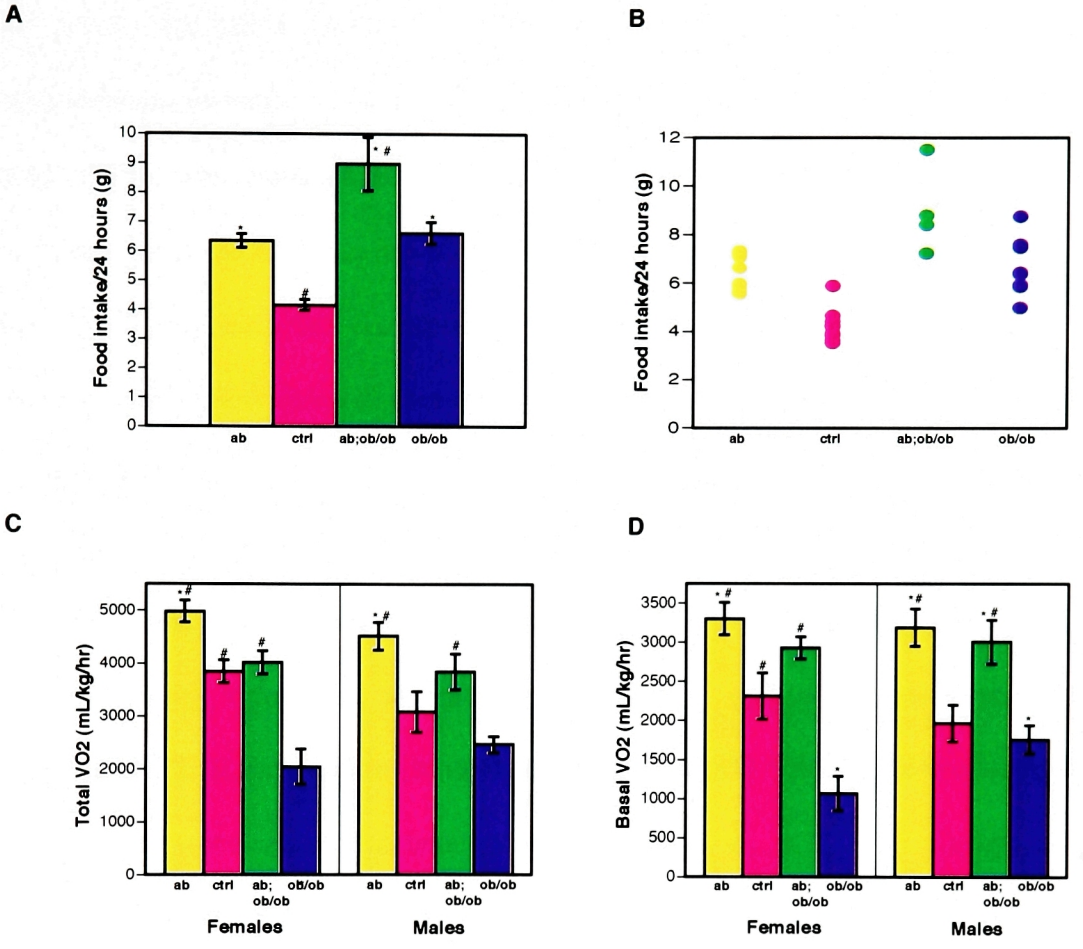


Figure 5.3 Increased energy expenditure, despite persistent hyperphagia, accounts for reduced adiposity in *ab^J/ab^J;ob/ob* and *ab^J/ab^J* mice. (A) Average daily food intake by group and (B) scatterplot of average daily food intake for individual mice in each group of *ob/ob* (blue), *ab^J/ab^J; ob/ob* (green), *ab^J/ab^J* (gold), and control (magenta) mice. Mice were acclimated in individual cages and 24 hour food consumption was measured for 8 consecutive days. Food intake for each mouse was averaged over the 8 days and these values were averaged for the mice in each group. Mice were 12-18 weeks of age. Food intake for males and females was indistinguishable and has been pooled. Error bars indicate SEM, $n \geq 4$ for each group. * $p < 0.05$ vs. lean control, $^\#$, $p < 0.05$ vs. *ob/ob*. (C) Total and (D) resting oxygen consumption of female and male *ob/ob* (blue), *ab^J/ab^J; ob/ob* (green), ab^J/ab^J (gold), and control (magenta) mice. 14-16 week old mice were placed in an indirect calorimeter and allowed 2 hours to acclimate to the new environment. Measurements were taken for 5 hours during the middle of the light cycle. The total VO2 is the average of these readings. The resting VO2 is the average of all readings that are one standard deviation below the total VO2, as these readings represent periods of inactivity. Error bars indicate SEM, $n \geq 3$ for each group. * $p < 0.05$ vs. lean control, $^\#$, $p < 0.05$ vs. *ob/ob*.

lipid-filled vacuoles, while those of *ab^J/ab^J; ob/ob* mice showed little or no vacuolation and were indistinguishable from those of wild-type mice (Figure 5.4a). The levels of liver triglyceride were substantially increased in *ob/ob* mice ($p < 0.005$ vs. all other groups), whereas triglycerides in *ab^J/ab^J; ob/ob* mice were reduced to levels comparable to lean controls (Figure 5.4b). As previously shown, triglyceride levels in lean *ab^J/ab^J* mice were reduced below those of lean controls (Figure 5.4b) (Miyazaki et al. 2000).

Palmitoleate and oleate in triglycerides and cholesterol esters are major constituents of very low density lipoprotein (VLDL) particles, which transport fatty acids from liver to adipose tissue and other sites. We assayed the rate of hepatic lipid export in mice injected with tyloxapol, an inhibitor of VLDL hydrolysis, permitting the measurement of VLDL production (Merkel et al. 1998). VLDL production was increased 2.4-fold in *ob/ob* mice relative to lean controls (Figure 5.4c, $p < 0.05$). Compared to *ob/ob* controls, VLDL production was reduced 3.6-fold in *ab^J/ab^J; ob/ob* mice (8.2 mg/dl/min vs. 2.3 mg/dl/min, $p < 0.02$) (Figure 5.4c).

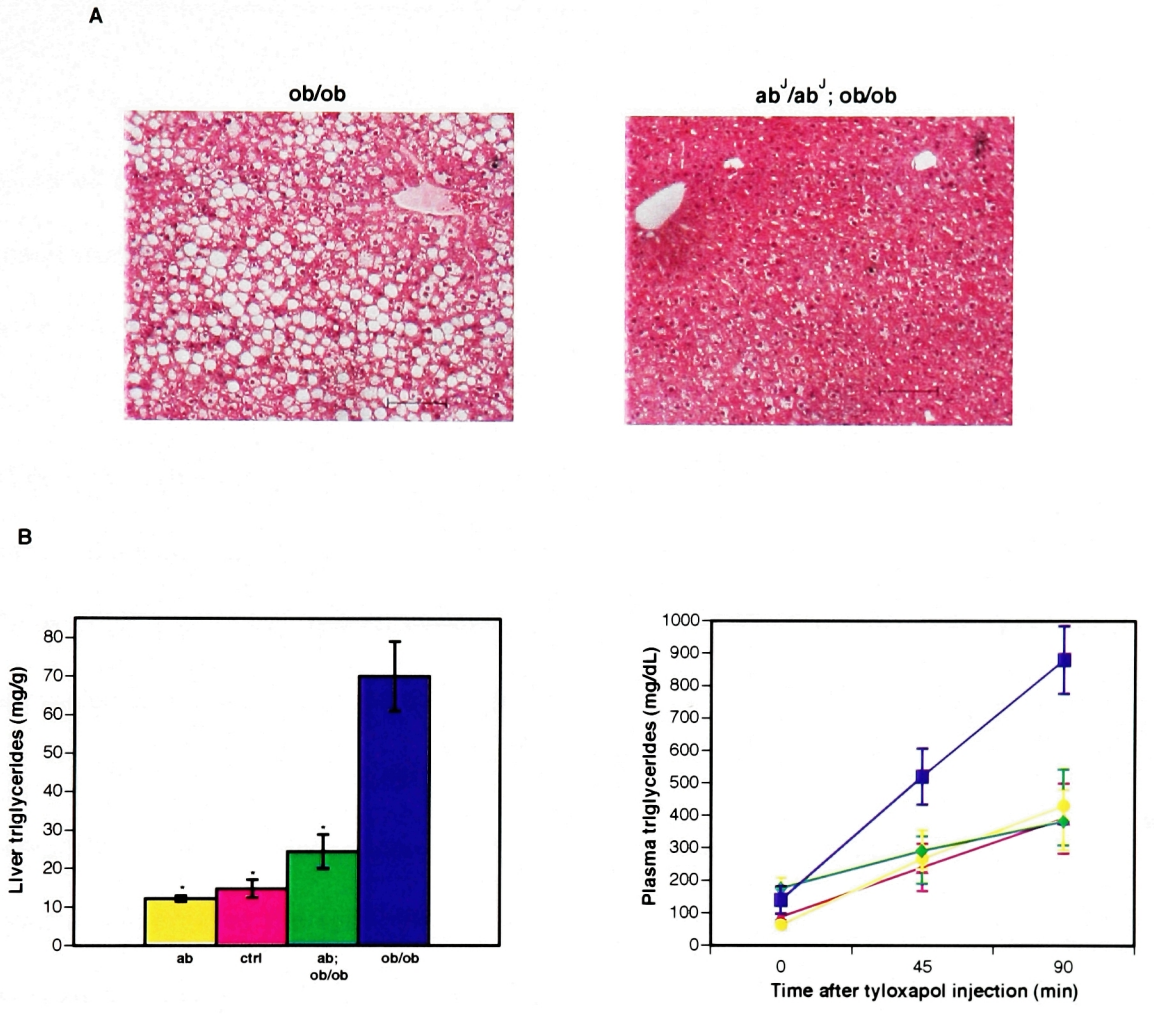


Figure 5.4 Reduced hepatic lipid storage and VLDL production in *ab^J/ab^J;ob/ob* mice. (A) Hematoxylin and eosin (H&E) stained liver sections from representative *ob/ob* and *ab^J/ab^J; ob/ob* mice. Images are 200x magnifications; scale bars denote 100 μm. (B) Liver triglyceride content of *ob/ob* (blue), *ab^J/ab^J; ob/ob* (green), *ab^J/ab^J* (gold), and control (magenta) mice. Levels were determined as described in Chapter 2. Error bars indicate SEM, $n \geq 4$ for each group. *, $p < 0.0005$ vs. *ob/ob*. (C) VLDL production in *ob/ob* (blue), *ab^J/ab^J; ob/ob* (green), *ab^J/ab^J* (gold), and control (magenta) mice. Mice fasted for 5 hours were injected with 0.5 mg/kg tyloxapol (Triton-1339) (Sigma, St. Louis, MO) via the tail vein as described in Chapter 2. Tail bleeds were done at 0, 45, and 90 min and plasma triglycerides were assayed using an enzymatic reagent. The slope of the line indicates the rate of VLDL production. Error bars indicate SEM, $n \geq 3$ for each group. For *ab^J/ab^J; ob/ob* and control, $p < 0.05$ vs. ob/ob. For *ab^J/ab^J*, $p = 0.07$ vs. *ob/ob*.

Discussion

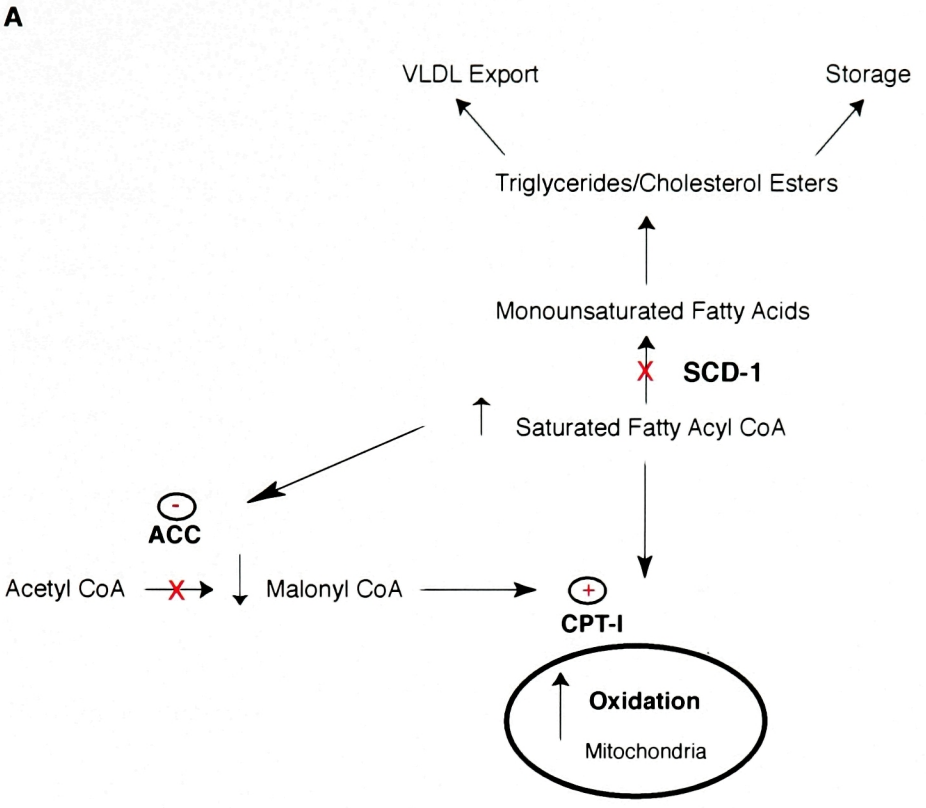
These data show that SCD-1 is required for the fully developed obese phenotype of *ob/ob* mice and suggest that a significant proportion of leptin's metabolic effects may result from inhibition of this enzyme. Mice with a targeted knockout of SCD-1 (SCD-$1^{-/-}$) have also been generated, and they resemble ab^J/ab^J mice in all respects (Miyazaki et al. 2001). These mice also have reduced fat mass and are resistant to increased adiposity after long-term consumption of a high-fat diet (Ntambi et al. 2002). SCD-$1^{-/-}$ mice also have enhanced glucose and insulin tolerance. Microarray analysis of gene expression in these mice detected increased expression of genes involved in fatty acid oxidation and decreased expression of lipogenic genes in SCD-$1^{-/-}$ mice (Ntambi et al. 2002).

The basis for the metabolic effects of SCD-1 deficiency is not known. One possibility is that in the absence of SCD-1, a reduced rate of triglyceride and VLDL synthesis increases the intracellular pool of saturated fatty acyl CoAs leading to an increase in fatty acid oxidation. Monounsaturated fats are necessary for normal rates of triglyceride and cholesterol ester synthesis, which are required for hepatic lipid storage and VLDL synthesis (Miyazaki et al. 2000). Saturated fatty acyl CoAs, but not monounsaturated fatty acyl CoAs, potently allosterically inhibit acetyl CoA carboxylase (ACC), reducing cellular levels of malonyl CoA (Volpe and Vagelos 1976; Lunzer et al. 1977; Nikawa et al. 1979). Malonyl CoA is required for fatty acid biosynthesis and also inhibits the mitochondrial carnityl palmityl transferase shuttle system, the rate-limiting step in the import and oxidation of fatty acids in mitochondria (McGarry et al. 1977). Reduced activity of SCD-1 may decrease adiposity by decreasing cellular levels of

malonyl CoA thereby reducing fatty acid biosynthesis and de-repressing fatty acid oxidation. Figure 5.5a shows a schematic of this potential mechanism. Moreover, preliminary data indicates that levels of saturated fatty acyl CoAs are, in fact, increased 3-fold or more in mice lacking SCD-1 (Figure 5.5b). Whether or not the increased saturated fatty acyl CoAs inhibit ACC and lead to reduced malonyl CoA and increased fatty acid oxidation remains to be tested.

These findings are similar to those described in mice lacking acetyl-CoA carboxylase 2, which also have increased fatty acid oxidation in skeletal muscle and a lean phenotype (Abu-Elheiga et al. 2001). The mechanism by which leptin increases fatty acid oxidation in liver may be similar to that in skeletal muscle in that both may operate by reducing ACC activity. However, in skeletal muscle, leptin, acting directly and indirectly via the CNS, inhibits ACC via α2 AMP-kinase, which phosphorylates and inhibits ACC (Minokoshi et al. 2002). Recent studies have shown that leptin stimulated fatty acid oxidation in liver and heart does not activate α2 AMP-kinase, indicating that other mechanisms may be involved, perhaps involving changes in SCD-1 activity (Atkinson et al. 2002; Lee et al. 2002).

Alternative mechanisms could also account for the metabolic effects of SCD-1 deficiency. Changes in SCD-1 activity could alter the levels of ligands for PPARα, PPARγ, or other nuclear hormone receptors. Changes in the ratio of saturated:unsaturated fatty acids in phospholipids can also alter membrane fluidity, which could affect signal transduction. The observed increase in energy expenditure associated with SCD-1 deficiency also suggests that uncoupling activity and/or futile cycles are induced. Reactive oxygen species are a byproduct of elevated oxidative metabolism and can



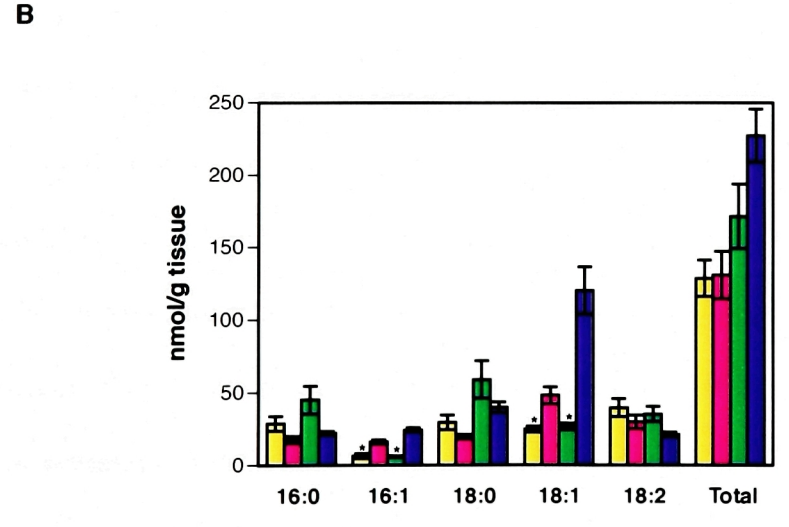


Figure 5.5 Basis for increased energy expenditure in mice lacking SCD-1. (A) Hypothetical mechanism for the metabolic effects of SCD-1 deficency. In the absence of SCD-1, synthesis of triglycerides and VLDL is blocked, leading to decreased hepatic lipid storage and export. A lack of SCD-1 causes an increase in the pool of saturated fatty acyl CoAs, which allosterically inhibit acetyl CoA carboxylase (ACC). Inhibition of ACC reduces levels of malonyl CoA, a metabolite which normally inhibits the mitochondrial carnityl palmitoyl shuttle system, the rate-limiting step in the import and oxidation of fatty acids in mitochondria. Decreased malonyl CoA thus de-represses fatty acid oxidation, leading to increased burning of fat. (B) Liver fatty acyl CoA levels were measured as decribed in Chapter 2 in *ob/ob* (blue), *ab^J/ab^J; ob/ob* (green), *ab^J/ab^J* (gold), and control (magenta) mice. Mice were scarificed in the mid-light cycle after a 6-10 hour fast. Error bars indicate SEM, $n \geq 3$ for each group, except control n= 2. * $p < 0.05$ *ab^J/ab^J* vs. lean control or *ab^J/ab^J; ob/ob* vs. *ob/ob* control.

increase cellular oxidative stress, thereby increasing the risk of cancer, atherosclerosis, and other diseases (Droge 2002). Future studies are necessary to determine whether increased oxidative stress is an unwanted side effect of SCD-1 deficiency.

The effects of leptin on SCD-1 in liver are likely to require central action, as mice lacking the leptin receptor in brain have enlarged, fatty livers, while livers of mice with a liver-specific knockout of the leptin receptor appear normal (Cohen et al. 2001). Leptin also reduces hepatic SCD-1 activity when administered intracerebroventricularly, at a dose that has no effect when delivered peripherally (E. Asilmaz, unpublished results). The CNS signals that modulate liver metabolism in response to leptin are unknown. SCD-1 deficiency also appears to modulate CNS pathways that regulate food intake, perhaps secondary to the increased oxygen consumption.

Leptin may also modulate the production of monounsaturated fatty acids in tissues other than liver. Down-regulation of SCD enzyme activity by leptin in other tissues, including brain, could contribute to some of the observed metabolic effects. Although SCD-1 is the only isoform normally expressed in liver, both SCD-1 and SCD-2, a similar enzyme, are expressed in most other tissues. While both enzymes catalyze the same reaction, their cellular distribution and substrate preferences may be different.

In summary, a deficiency of SCD-1 ameliorates the obesity of *ob/ob* mice and completely corrects the hypometabolic phenotype of leptin deficiency. These findings suggest that leptin-specific down regulation of SCD-1 is an important component of the novel metabolic response to leptin and suggests that inhibition of SCD-1 could be of benefit for the treatment of obesity, hepatic steatosis, and other metabolic disorders.

Chapter 6: Role for Stearoyl-CoA Desaturase-1 in Fatty Liver Disease

Introduction

Hepatic steatosis, or fatty liver, is the earliest manifestation of both alcoholic and nonalcoholic liver disease. Both types of fatty liver disease are marked by the histological presence of steatosis and a mixed inflammatory cell infiltrate, and as disease worsens, by the appearance of hepatocyte necrosis and fibrosis (Angulo 2002). In fact, the two types of disease are histologically indistinguishable, and the differential diagnosis is largely based on the presence or absence of alcohol abuse (Yu and Keeffe 2002). Fatty livers are particularly susceptible to endotoxin-mediated damage, which in turn, accelerates the progression of disease to steatohepatitis, fibrosis, cirrhosis, and end-stage liver disease. (Yang et al. 1997; Diehl 2001; Yang et al. 2001). Nonalcoholic fatty liver disease (NFLD) is now recognized as the most common form of liver disease in the United States, and alcoholic fatty liver disease is a major cause of morbidity and mortality (Menon et al. 2001; McCullough 2002). Furthermore, both forms of disease are largely untreatable. Thus, understanding the molecular basis for fatty liver disease is required to develop effective treatments.

While alcoholic fatty liver disease is, by definition, a result of chronic alcohol abuse, NFLD has a diverse set of etiologies. Both obesity and type 2 diabetes mellitus markedly increase the risk and severity of NFLD (Silverman et al. 1990; Wanless and Lentz 1990; Bellentani et al. 2000). While NFLD is estimated to affect 10-24% of the population, the prevalence among obese individuals has been estimated to be as high as 74% (Angulo 2002). Both types of disease are characterized, in their early stages, by the

presence of hepatic steatosis. Lipid can accumulate in hepatocytes due to either increased fatty acid uptake or biosynthesis or decreased fatty acid export or oxidation (Koteish and Diehl 2001).

Alcoholic fatty liver was originally thought to result from the generation of NADH during ethanol oxidation, which then inhibits NAD^+-dependent enzymes of the citric acid cycle and ß–oxidation, thereby inhibiting hepatic fatty acid oxidation (Grunnet and Kondrup 1986; Crabb 1993). However, increased lipogenesis is being increasingly appreciated as underlying alcoholic fatty liver (Lieber et al. 1966; Muramatsu et al. 1981; Carrasco et al. 2001). A number of studies have demonstrated increased RNA levels and enzymatic activity of lipogenic enzymes in ethanol fed rodent models (Joly et al. 1973; Arakawa et al. 1975; Muramatsu et al. 1981; You et al. 2002).

Increased rates of hepatic lipogenesis have also been demonstrated in obese rodents and humans, who are more prone to NFLD (Godbole and Yotk 1978; Kaplan and Leveille 1981; Diraison et al. 2002).

Stearoyl-CoA desaturase-1 (SCD-1) is the rate-limiting enzyme in the conversion of saturated to monounsaturated fatty acids (Ntambi 1995). SCD-1 is required for normal rates of hepatic triglyceride and cholesterol ester biosynthesis (Miyazaki et al. 2000). SCD-1 RNA levels and enzymatic activity are increased in *ob/ob* mice, and SCD-1 plays a role in leptin-mediated weight loss, as *ob/ob* mice lacking SCD-1 (ab^J/ab^J; *ob/ob*) demonstrate a significant correction of obesity (Chapter 5)(Cohen et al. 2002). ab^J/ab^J; *ob/ob* mice also have a dramatic correction in the hepatic steatosis typical of *ob/ob* mice. This suggested that SCD-1 may play a more general role in fatty liver disease. Here, we

examine the role of SCD-1 in alcoholic fatty liver disease and in NFLD secondary to lipodystrophy.

Results

SCD-1 and alcoholic fatty liver disease

To explore the role of SCD-1 in alcohol-induced fatty liver, we studied *asebia* mice (ab^J/ab^J), an inbred, naturally occurring mutant with a mutation in SCD-1 (Gates and Karasek 1965; Zheng et al. 1999). After an acclimation period, ab^J/ab^J and littermate control mice ($ab^J/+$ or +/+) were given a 10% ethanol solution as their sole source of fluid. Mice will consume alcohol at this concentration, and this treatment induces fatty liver in a number of mouse models (Furuno et al. 1975; Wood 1976; Watabiki et al. 2000).

ab^J/ab^J and littermate controls were sacrificed after 3 and 5 weeks of alcohol consumption. As an additional control, age-matched mice of each genotype that were not exposed to alcohol were also studied. After 3 weeks of alcohol intake, livers from both ab^J/ab^J and control mice appeared grossly normal. In addition, liver triglyceride content was equivalent to that of untreated control mice (Fig. 6.1a). After 5 weeks of ethanol, however, littermate controls developed grossly steatotic livers, while livers from ab^J/ab^J mice appeared normal. Quantitation of liver triglycerides revealed a 2-fold increase in littermate controls mice relative to ab^J/ab^J (17.4 mg/g vs. 8.6 mg/g, $p = 0.0009$) (Figure 6.1a). Furthermore, even after five weeks of alcohol consumption, triglyceride levels in ab^J/ab^J mice remained indistinguishable from mice not given alcohol.

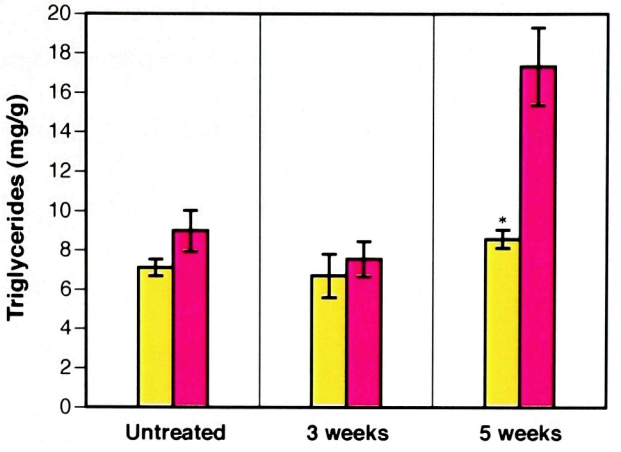
In order to confirm that the absence of fatty liver disease in ab^J/ab^J mice was not due to reduced alcohol consumption, we measured alcohol intake. Surprisingly, ab^J/ab^J mice actually consumed more alcohol than littermate controls. To quantitate alcohol consumption more exactly, 5 male ab^J/ab^J and 5 littermate controls were individually caged, and food and alcohol intake were recorded weekly. ab^J/ab^J mice consumed more food than littermate controls, at all time points (Figure 6.2b). While they also consumed more alcohol within the first few weeks, alcohol intake was comparable between the two groups thereafter (Figure 6.2b). Thus, despite consuming more food and an equivalent amount of alcohol ab^J/ab^J mice remained resistant to the development of alcoholic fatty liver.

SCD-1 and nonalcoholic fatty liver disease secondary to lipodystrophy

Transgenic overexpression of nuclear sterol regulatory element-binding protein-1c (*aP2-SREBP-1c tg*) in white adipose tissue produces mice with a near complete absence of adipose tissue, along with marked hepatic steatosis, insulin resistance, and hyperglycemia (Shimomura et al. 1998a). The livers in these mice closely resemble those of *ob/ob* mice, and in both cases, treatment with physiological doses of leptin for 12 days corrects the hepatic steatosis, along with the hyperglycemia and hyperinsulinemia (Chapter 4) (Shimomura et al. 1999c). A number of human lipodystrophy syndromes have also been described, and treatment of these patients with leptin also reverses hepatic steatosis and insulin resistance (Oral et al. 2002; Petersen et al. 2002).

To study the role of SCD-1 in the fatty liver disease of lipodystrophy, ab^J/ab^J mice were crossed to *aP2-SREBP-1c tg* mice to generate lipodystrophic mice lacking

A



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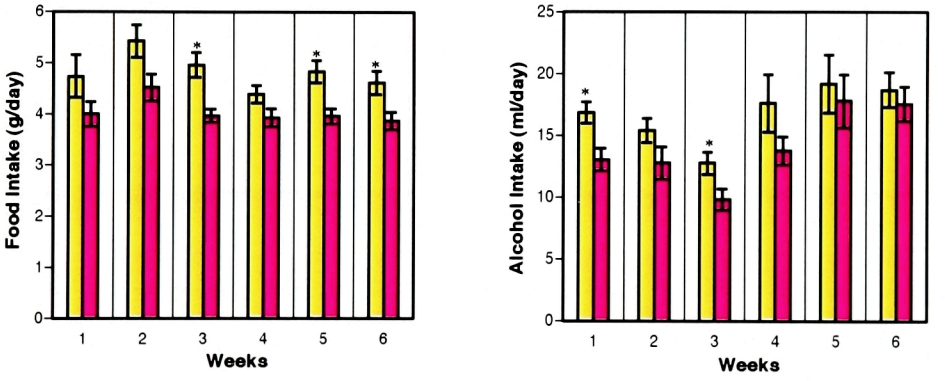

Figure 6.1 Resistance to alcoholic fatty liver in mice lacking SCD-1. (A) 5-7 week old, male and female ab^J/ab^J (gold) and littermate control mice (magenta) were offered a 10% EtOH solution for one week. After this acclimation period (time 0), mice were provided with10% EtOH as the only liquid source. Groups of animals were sacrificed after 3 and 5 weeks of alcohol consumption. As a control, similarly aged mice, that were not exposed to alcohol were also studied (untreated). Error bars indicate SEM, $n \geq 3$ for each group. *, $p = 0.0009$. (B) 8-10 week old ab^J/ab^J and littermate control mice were individually caged and given a few days to acclimate to a 10% EtOh solution. Then, regular drinking water was removed (time 0) and weekly food and alcohol intake were recorded. Error bars indicate SEM, $n = 5$ for each group. *, $p < 0.05$ ab^J/ab^J vs. control.

SCD-1 (*ab^J/ab^J;aP2-SREBP-1c tg*). Analysis of these mice confirmed that *aP2-SREBP-1c tg* mice had massively enlarged steatotic livers. Livers from *ab^J/ab^J;aP2-SREBP-1c tg* mice, on the other hand, remained enlarged, but were no longer grossly steatotic (Figure 6.2a). Histological sections showed hepatic vacuolation consistent with fatty liver disease in *aP2-SREBP-1c tg* mice, while *ab^J/ab^J;aP2-SREBP-1c tg* sections showed a nearly normal histological appearance (Figure 6.2b). Measurement of liver triglyceride levels revealed a 4-fold increase in triglyceride levels in *aP2-SREBP-1c tg* livers relative to double mutant livers (34.0 ± 8.9 mg/g vs. 8.2 ± 1.2 mg/g, $p = 0.02$) (Figure 6.2c). Triglyceride levels in *ab^J/ab^J;aP2-SREBP-1c tg* livers were comparable to those in wild-type littermate controls.

Discussion

The data presented here indicate that the absence of SCD-1 can not only correct the fatty liver of obesity (Chapter 5), but can also block the development of alcoholic fatty liver disease and nonalcoholic fatty liver disease associated with lipodystrophy. Elevated lipogenesis has been postulated to cause both types of fatty liver disease, and mice lacking SCD-1 have decreased rates of hepatic triglyceride production (Miyazaki et al. 2000). Reduced hepatic fatty acid oxidation can also contribute to fatty liver disease. Evidence suggests that the absence of SCD-1 indirectly leads to increased liver fatty acid oxidation (Cohen et al. 2002; Ntambi et al. 2002). Enhanced fatty acid oxidation could also underlie the resistance to hepatic steatosis in mice lacking SCD-1. Elevated fatty acid oxidation can be associated with increased levels of reactive oxygen species (Droge

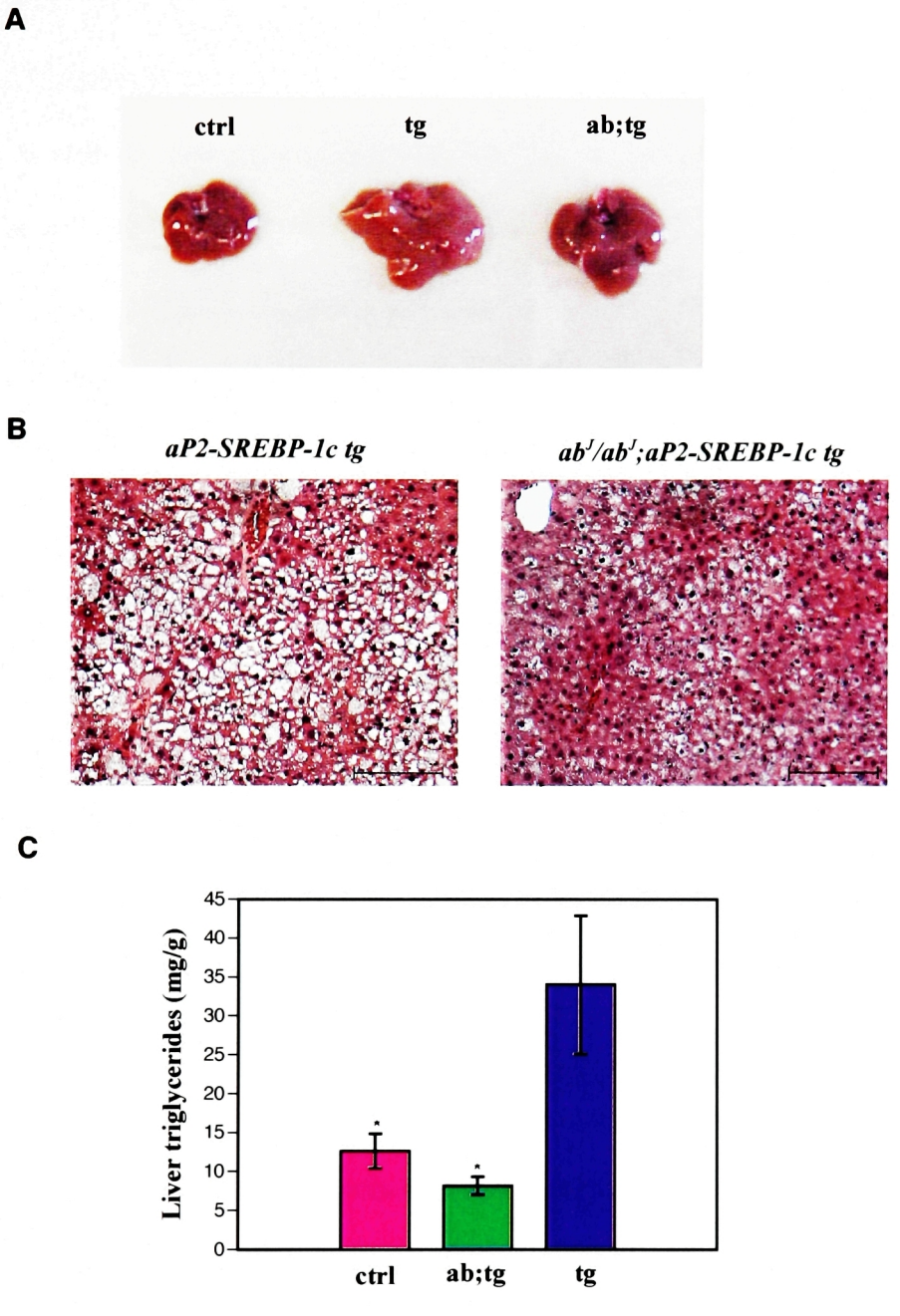

Figure 6.2 Resistance to fatty liver associated with lipodystrophy in mice lacking SCD-1. (A) Photograph of freshly dissected livers from mice with the genotypes *+/+;nontg* (ctrl), *ab^J/+;aP2-SREBP-1c tg* (tg), and *ab^J/ab^J;aP2-SREBP-1c tg* (ab;tg). (B) Hematoxylin and eosin stained liver sections from a representative *aP2-SREBP-1c tg* and an *ab^J/ab^J;aP2-SREBP-1c tg* mouse. Images are 200x magnifications; scale bars denote 100 μm. (C) Liver triglyceride content of control (*+/+;nontg* and *ab^J/+;nontg*) (magenta) *ab^J/ab^J;aP2-SREBP-1c tg* (green), and transgenic (*ab^J/+;aP2-SREBP-1c tg* and *+/+;aP2-SREBP-1c tg*) (blue). Error bars indicate SEM, $n \geq 4$ for each group. *, $p < 0.05$ vs transgenic.

2002). Since increased (ROS) contribute to fatty liver disease as well as to other disease processes, it will be important to test whether this is a sequella of SCD-1 deficiency (Pessayre et al. 2002).

Increased lipogenesis is a recognized feature of alcoholic fatty liver (Lieber et al. 1966; Muramatsu et al. 1981; Carrasco et al. 2001). Expression analysis of liver RNA from alcohol fed mice found a 3-fold increase in SCD-1 RNA levels (data not shown). Real-time quantitative PCR confirmed this increase in SCD-1 expression (data not shown). A number of studies have demonstrated increased RNA levels and enzymatic activity of lipogenic enzymes in ethanol fed rodent models (Joly et al. 1973; Arakawa et al. 1975; Muramatsu et al. 1981). A recent study showed that the levels of SREBP target genes, including SCD-1, are upregulated in alcoholic fatty liver, due to increased levels of cleaved, nuclear SREBP (You et al. 2002). In addition, ethanol-fed rodents have a dramatic increase in hepatic levels of palmitoleic and oleic acid, the products of SCD-1 (Tsukamoto et al. 1984). Further study is required to determine whether ab^J/ab^J mice remain resistant to alcoholic fatty liver disease after more chronic ethanol administration. In addition, longer exposure to alcohol is associated with a progression to hepatitis, fibrosis, and ultimately cirrhosis, and it will be interesting to see whether mice lacking SCD-1 are also resistant to further liver damage.

Lipodystrophic mice also demonstrate increased hepatic lipogenesis, due to increased expression of fatty acid biosynthetic enzymes, including SCD-1 (Shimomura et al. 1998a). Leptin administration corrects diabetes and hepatic steatosis, and is associated with a normalization in the expression of SCD-1 and other lipogenic molecules (E.

Asilmaz, unpublished results) (Shimomura et al. 1999c). Since these mice lack adipose tissue, they also deposit triglyceride in a number of other peripheral tissues, such as muscle, heart, kidney, and spleen (Garg 2000; Reitman et al. 2000). *ab^J/ab^J;aP2-SREBP-1c tg* mice appear to be resistant to triglyceride accumulation in these tissues as well (data not shown). While hepatic steatosis was prevented, livers remain grossly enlarged in double mutant animals, and the cause for this remains unknown. In addition, it is not yet clear whether *ab^J/ab^J;aP2-SREBP-1c tg* mice have any improvement in insulin resistance or hyperglycemia.

Mice with transgenic overexpression of nuclear sterol regulatory element-binding protein-1a (*PEPCK-SREBP-1a tg*) in liver also have massively enlarged livers, engorged with lipid (Shimano et al. 1996). These mice have increased expression of genes involved in the entire program of monounsaturated fatty acid biosynthesis (Shimomura et al. 1998b). In addition, A^y mice, which develop severe adult onset obesity, also have increased hepatic lipogenesis and NFLD (Yen et al. 1976). Both of these mutants have been crossed to *ab^J/ab^J* mice, and studies are underway to examine whether the absence of SCD-1 can prevent fatty liver disease in these models.

In the absence of SCD-1, two different forms of fatty liver disease could be largely prevented. The incidence of NFLD has been steadily rising in parallel with the rise in obesity and diabetes. At present, there is no effective treatment other than correction of the underlying condition (Angulo 2002). Based on these results, the inhibition of SCD-1 may be of benefit in treating fatty liver disease.

Chapter 7: Role for Insulin-like Growth Factor Binding Protein-2 in Leptin-mediated Weight Loss

Introduction

Leptin has metabolic actions that are distinct from its effects on food intake (Levin et al. 1996). Transcriptional profiling of *ob/ob* liver during a time course of weight reduction demonstrated a program of gene expression that is specific to leptin-mediated weight loss (Chapter 4). Cluster analysis identified several groups of genes whose expression was uniquely modulated by leptin as compared to pair-feeding. These clusters were broadly divided into two classes: (i) genes with increased expression that were preferentially reduced with leptin treatment and (ii) genes with decreased expression that were preferentially induced with leptin treatment.

To prioritize the first class of genes for functional study, an algorithm was developed to identify and rank genes in *ob/ob* liver that were specifically reduced by leptin administration. This list served as a molecular signature of leptin action and could contain genes that are functionally important in energy homeostasis. SCD-1, the gene that ranked at the top of this list, was found to play an important role in obesity and fatty liver disease (Chapters 5 and 6)(Cohen et al. 2002; Ntambi et al. 2002).

While *ob/ob* mice lacking SCD-1 showed a marked improvement in adiposity and other components of the obese phenotype, the absence of this gene alone did not completely correct the abnormalities of *ob/ob* mice. This suggested that other alterations in gene expression, typical of *ob/ob* mice, remained and contributed to the residual

phenotype in these mice. To identify additional genes involved in leptin's metabolic effects, we used an algorithm to prioritize the second class of genes, those that were reduced in *ob/ob* liver and specifically induced by leptin. Insulin-like growth factor binding protein-2 (IGFBP-2) ranked at the top of this list and studies on the role of this molecule in leptin-mediated weight loss are presented here.

Results

Modulation of IGFBP-2 RNA levels

The algorithm described in Chapter 5 (and more fully in Chapter 2) was modified to rank genes based on the extent to which their expression was (1) decreased in *ob/ob* liver compared to wild type (2) induced by leptin treatment, and (3) different between leptin treatment and pair feeding. Genes with the lowest scores are those most strongly leptin-regulated. Such genes may be functionally involved in the metabolic effects of leptin. The results of this analysis are shown in Table 7.1.

The list of leptin-induced genes included a diverse number of functional categories. Unlike the list of leptin repressed genes (Table 5.1), the majority of which function in lipid and carbohydrate metabolism, nearly half of the leptin-induced genes are secreted. The identification of such factors may reflect a normalization of impaired liver function, secondary to leptin-deficiency, insulin resistance, and hepatic steatosis. Alternatively, some of these genes could also be directly involved in the unique program of leptin-mediated weight loss.

The genes in Table 7.1 included a number of molecules that are components of the growth hormone (GH) / insulin-like growth factor (IGF) pathway. Growth hormone is

Leptin-induced Gene Expression in *ob/ob* Liver

Accesion Number	Name	Function	Score
L05439	insulin-like growth factor binding protein 2	Binding Protein	4.50
L11333	carboxyesterase	Detoxification	4.50
U29762	albumin gene D-Box binding protein	DNA Binding	4.50
X64263	apolipoprotein A-I	Lipid Metabolism	6.00
M16358	major urinary protein IV	Secreted	6.00
X00496	Ia-associated invariant chain	Immune	8.00
U22516	angiogenin precursor	Angiogenesis	9.00
U66900	acid labile subunit	Binding Protein	10.00
M57891	complement component C2	Inflammatory	11.00
L11613	20S proteasome subunit Lmp2	Protein Turnover	13.50
M75721	alpha-1 protease inhibitor 1	Secreted	13.50
M16359	major urinary protein III	Secreted	14.50
X70533	corticosteroid-binding globulin	Binding Protein	16.00
X03208	group 1 for major urinary protein	Secreted	16.50
J00479	Ig gamma2a-b	Immune	17.00
V01527	MHC class II antigen, I-A-beta	Immune	18.00
U06924	stat1	Signal Transduction	19.50
L29006	solute carrier family 7	Transport	21.00
X70398	P311	Unknown	22.00
U19119	interferon inducible protein 1	Inflammatory	22.50
U44088	TDAG51	Signal Transduction	23.00
X95685	17-beta-hydroxysteroid dehydrogenase type II	Steroid Metabolism	24.50
D16492	mannan-binding lectin serine protease 1	Secreted	25.00
X04653	Ly-6 alloantigen	Membrane Protein	25.00
U22031	20S proteasome subunit Lmp7	Protein Turnover	25.50
Y00964	beta-hexosaminidase	Secreted	25.75
U28937	regucalcin	Signal Transduction	26.25
D45850	17-beta-hydroxysteroid dehydrogenase 5	Steroid Metabolism	27.00
L07645	histidine ammonia lyase	Amino Acid Metabolism	27.00
X70391	inter-alpha trypsin inhibitor, heavy chain 1	Secreted	28.00
AA002504	est, similar to human hyaluronan binding protein 2	Unknown	28.50
U00674	syndecan 2	Secreted	28.50
J04766	plasminogen	Clotting	29.50
X04480	preproinsulin-like growth factor IA	Growth	31.00
M81447	connexin 32	Structural	32.50
U36993	cytochrome P450, 7b1	Steroid Metabolism	33.50
X58426	hepatic triglyceride lipase	Lipid Metabolism	34.50
U15636	GTP binding protein GTP2	Signal Transduction	37.00
AA120109	est, similar to rat ERG2	Unknown	38.50

Table 7.1 Leptin-induced gene expression in *ob/ob* Liver. Genes were ranked based on the extent to which their expression was (1) decreased in *ob/ob* liver compared to wild-type, (2) induced by leptin treatment, and (3) maximally different between leptin treatment and pair-feeding. Genes with the lowest scores are most strongly leptin-induced.

secreted into the bloodstream by the pituitary gland and stimulates the production of IGF-I, particularly from the liver. IGF-I then circulates in a complex with any of a number of binding proteins, known as insulin-like growth factor binding proteins (IGFBPs), which modulate IGF-I action. IGF-I has pleiotropic actions on multiple target tissues, generally stimulating cell growth and division (Le Roith et al. 2001). The list of leptin-induced genes included prepro-IGF-Ia itself, insulin-like growth factor binding protein-2 (IGFBP-2) and acid labile subunit (ALS), which bind IGF-I in the circulation, and three different major urinary proteins, the levels of which are positively regulated by GH (Knopf et al. 1983; Clemmons 1998).

One of the three genes sharing the top rank for being most strongly leptin-induced was IGFBP-2, a member of a family of IGF binding proteins, which has endocrine functions by modulating serum IGF half-life and transport, as well as autocrine and paracrine functions by blocking or enhancing the availability of IGFs to bind their receptors (Binkert et al. 1989; Schuller et al. 1994; Ranke and Elmlinger 1997). Northern blot analysis showed that IGFBP-2 RNA levels were undetectable in *ob/ob* mice, and were induced to wild-type levels with leptin replacement (Figure 7.1a). Pair-feeding, on the other hand, had a negligible effect on IGFBP-2 expression. Leptin also specifically induced ALS, which circulates as a ternary complex with IGFs and IGFBP-3, stabilizing this complex and thereby increasing the half-life of IGFs (Boisclair et al. 1996). With the exception of IGFBP-2 and ALS, based on microarray analysis, the other known IGFBPs were not found to be leptin-induced.

To gain further insight into the role of IGFBP-2, its RNA levels were also measured in a number of other rodent models of obesity (Figure 7.1b). IGFBP-2 was expressed at equivalent levels in livers of C57Bl/6J and AKR/J, two inbred strains of wild-type mice. IGFBP-2 was markedly suppressed in mice lacking leptin or totally resistant to its actions (*ob/ob*, db^{Kls}/db^{Kls}, and db^{3J}/db^{3J}). In addition, IGFBP-2 expression was drastically decreased in moderately obese transgenic mice, constitutively expressing a low level of leptin (*ob/ob tg*)(Ioffe et al. 1998). Interestingly, IGFBP-2 levels were reduced far less in A^y and New Zealand obese (NZO) mice, which manifest partial leptin resistance (Halaas et al. 1997). IGFBP-2 expression was also measured in each of these models following a 48 hour fast (Figure 7.1b). In every case, fasting led to a marked upregulation in IGFBP-2, confirming previous studies in wild-type rodents (Tseng et al. 1992). The modulation of IGFBP-2 levels by leptin, its alteration in numerous rodent obesity models, and its regulation by fasting support a role for this molecule in energy homeostasis.

Attenuated response to leptin in *ob/ob* mice lacking IGFBP-2

In order to examine the functional role of IGFBP-2 induction by leptin, we studied IGFBP-2 knockout mice. Aside from reduced spleen size and increased liver size, IGFBP-2 null mice have no obvious abnormalities, possibly due to redundancy within the IGFBP family (Wood et al. 1993; Pintar et al. 1995; Wood et al. 2000). To test whether IGFBP-2 induction is important in leptin-mediated weight loss, we crossed *IGFBP-2*$^{-/-}$ mice to *ob/ob* mice to generate double mutant animals. These mice were then treated with leptin, and their response was compared to that of *ob/ob* mice. We hypothesized that if

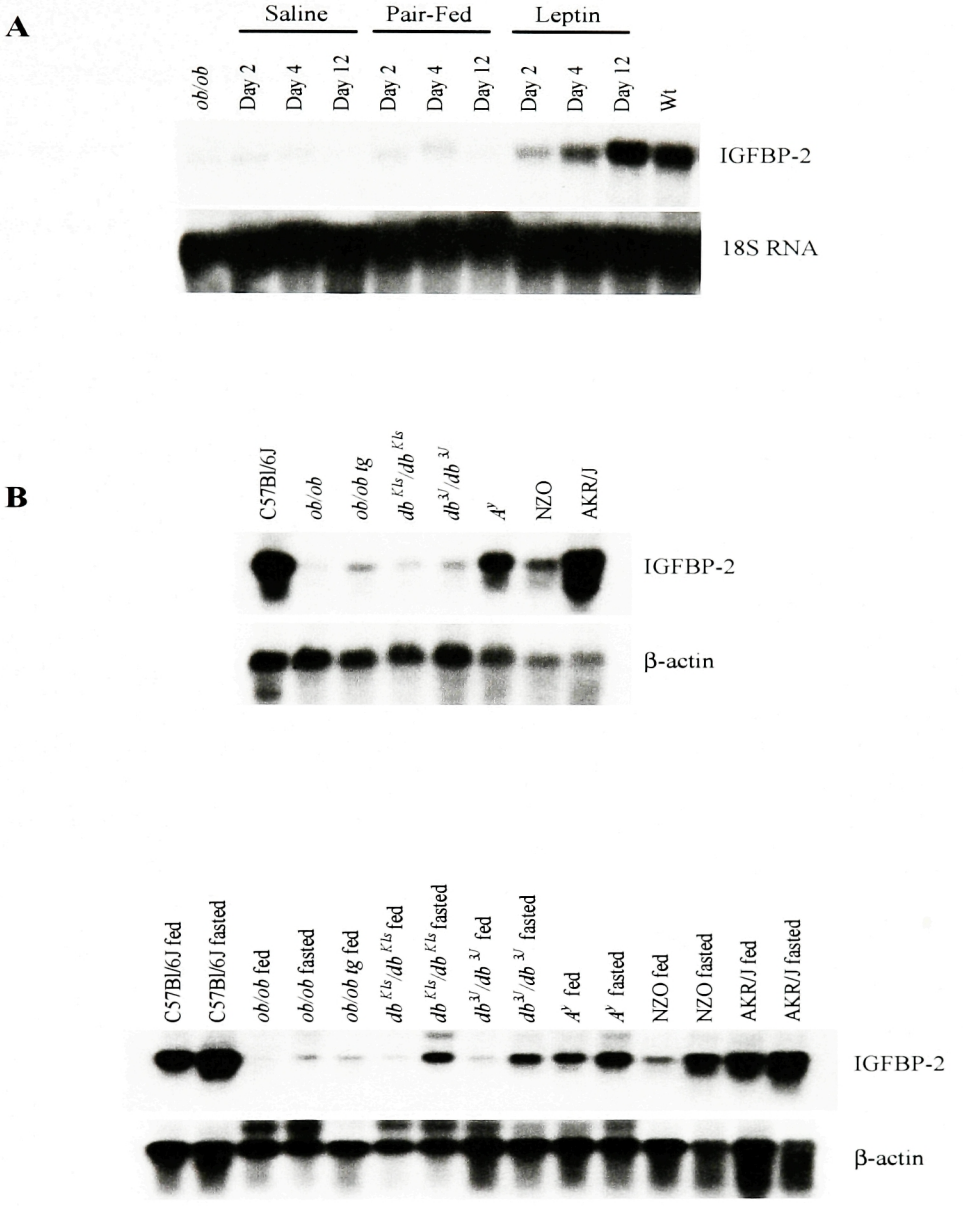

Figure 7.1 Regulation of IGFBP-2 RNA levels by leptin and nutritional status. Northern blots of 10 μg of liver RNA were hybridized with radioactively labeled probes specific for IGFBP-2, 18S RNA, or β-actin. IGFBP-2 RNA levels in (A) time course experiment and (B) different mouse models of obesity in either the fed state or after a 48 hour fast.

IGFBP-2 is involved in leptin's metabolic effects, then *ob/ob* mice lacking IGFBP-2 might have a diminished response to leptin.

After ten days of subcutaneous leptin administration, *IGFBP-2*$^{-/-}$*;ob/ob* and *ob/ob* mice showed marked differences in the amount of weight loss (Figure 7.2a). *ob/ob* mice lost nearly twice as much weight as *IGFBP-2*$^{-/-}$*;ob/ob* mice (28% vs. 15% weight loss, $p = 0.008$), with significant differences between these mice detectable as early as five days after initiating leptin treatment. This is consistent with the time course of IGFBP-2 induction in leptin-treated *ob/ob* mice (Figure 7.1a). Leptin also had a significantly more potent anorectic effect in *ob/ob* than on *IGFBP-2*$^{-/-}$*;ob/ob* mice (Figure 7.2b).

These data suggest that leptin can reach higher circulating levels and/or produce more potent effects in obese mice capable of inducing IGFBP-2. While leptin levels in both groups of mice were close to the physiological range, levels in *ob/ob* mice were 50% greater than those in IGFBP-2$^{-/-}$;*ob/ob* mice (17.2 ng/ml vs. 11.9 ng/ml, $p = 0.04$) (Figure 7.2d). In addition, as shown before, leptin completely corrected hyperglycemia in *ob/ob* mice (glucose: 91.5 mg/dl) (Halaas et al. 1995; Pelleymounter et al. 1995). *IGFBP-2*$^{-/-}$*;ob/ob* mice, on the other hand, showed a more modest reduction in hyperglycemia, though glucose levels were substantially higher in these mice than in *ob/ob* controls (glucose: 174 mg/dl, $p = 0.01$ vs. leptin treated *ob/ob*) (Figure 7.2c).

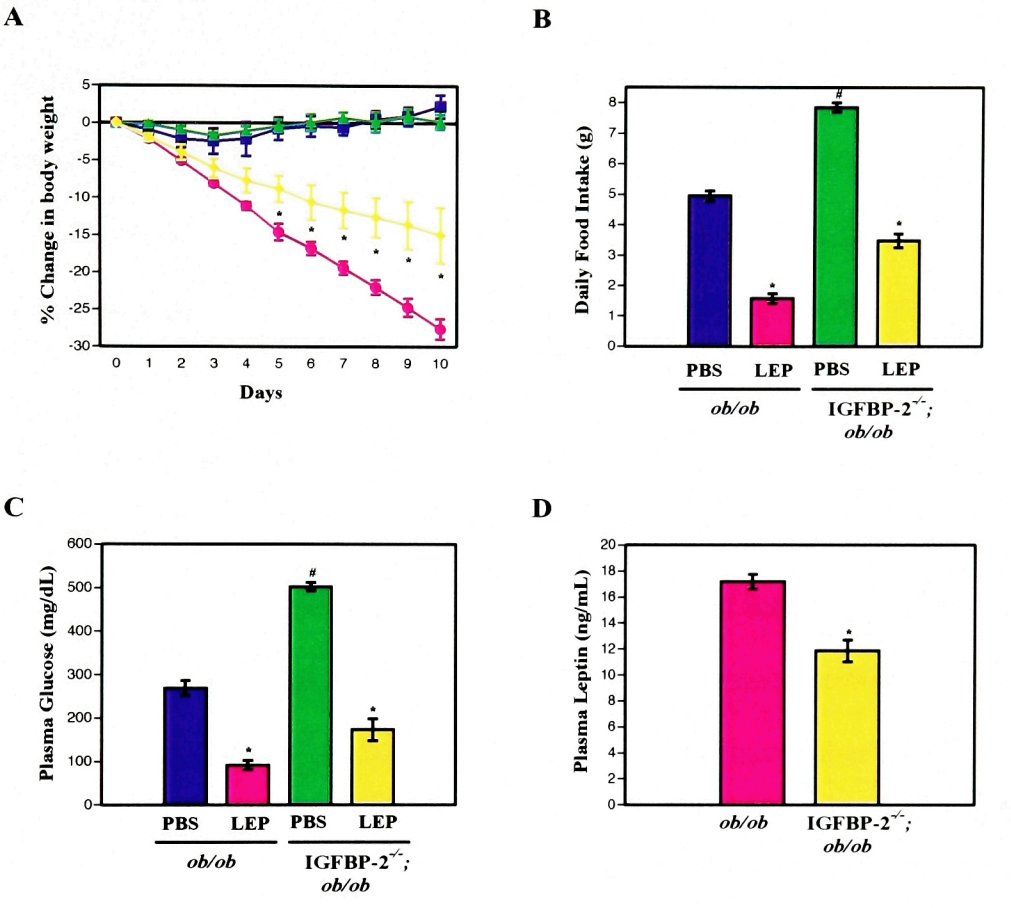

Figure 7.2 Attenuated response to leptin in *IGFBP2*$^{-/-}$;*ob/ob* mice. *ob/ob* and *IGFBP2*$^{-/-}$;*ob/ob* mice were treated with subcutaneous pbs or leptin (4.8 μg/day) for 10 days. (A) percent change in body weight, *, $p < 0.05$ *ob/ob* vs. *IGFBP2*$^{-/-}$;*ob/ob* leptin treated. (B) daily food intake averaged over days 3-10 of treatment. *, $p < 10^{-5}$ pbs vs. leptin; $^{\#}$, $p < 10^{-5}$ *ob/ob* vs. *IGFBP2*$^{-/-}$;*ob/ob* (C) plasma glucose levels, *, $p < 10^{-5}$ pbs vs. leptin; $^{\#}$, $p < 10^{-5}$ *ob/ob* vs. IGFBP2$^{-/-}$;*ob/ob* and (D) plasma leptin, *, $p < 0.05$ *ob/ob* vs. *IGFBP2*$^{-/-}$;*ob/ob*. $n \geq 4$ for each group, error bars indicate SEM.

Discussion

The data presented here indicate that IGFBP-2 is necessary for the full complement of leptin's metabolic effects. Double mutant mice lost significantly less body weight than *ob/ob* counterparts. *IGFBP-2*$^{-/-}$*;ob/ob* mice were also observed to be leptin resistant in a separate time course experiment (data not shown). Although the *IGFBP-2*$^{-/-}$*;ob/ob* mice were older than the *ob/ob* mice studied here, in seaprate experiments older *ob/ob* mice also lost approximately 30% of their body weight following leptin therapy (as opposed to 15% in *IGFBP-2*$^{-/-}$*;ob/ob* mice). In addition, *IGFBP-2*$^{-/-}$*;ob/ob* mice have less marked reductions in hyperglycemia. This could point to a specific function of IGFBP-2, or may simply be due to the more modest weight loss in these mice. Further study, in inbred mice is required to determine whether double mutant mice have other phenotypic alterations relative to *ob/ob* mice.

Despite the lack of any dramatic phenotype in IGFBP-2 knockout mice, perhaps due to functional redundancy, data from transgenic mice and obese humans support a role for this molecule in body weight physiology (Wood et al. 1993; Pintar et al. 1995; Wood et al. 2000). Transgenic mice expressing IGFBP-2 under the control of the CMV promoter expressed IGFBP-2 in every tissue, but liver, and had a 3-fold increase in serum IGFBP-2 levels (Hoeflich et al. 1999). These mice developed normally, but from weaning onward showed a significant reduction in body weight (approximately 10%) at all ages (Hoeflich et al. 1999) (Schneider et al. 2000). In addition, transgenic mice had reduced glucose levels relative to non-transgenic controls. When outbred mice were selectively bred to generate lines of mice with either low or high body weight, low body

weight was associated with elevated hepatic IGFBP-2 expression, while high body weight was associated with reduced hepatic IGFBP-2 expression (Hoeflich et al. 1998). IGFBP-2 levels were also suppressed in obese humans, and this suppression was accentuated by the concomitant presence of diabetes (Nam et al. 1997; Frystyk et al. 1999).

The findings in leptin treated *IGFBP-2*$^{-/-}$*;ob/ob* mice are highly similar to those in pair-fed *ob/ob* mice. Since *ob/ob* mice have undetectable IGFBP-2 expression, which is not induced at all by pair-feeding, the induction of IGFBP-2 upon leptin treatment could be responsible for many of the differences observed between leptin treated and pair-fed mice. Pair-fed mice also lose significantly less weight than leptin-treated *ob/ob* mice and show a less dramatic correction of the diabetic phenotype (Levin et al. 1996). Since pair-fed mice lose both fat mass and lean mass, whereas leptin treated mice only lose fat mass, body composition analysis of leptin treated *IGFBP-2*$^{-/-}$*;ob/ob* mice is necessary to determine which body compartments contribute to their weight loss (Halaas et al. 1995; Pelleymounter et al. 1995).

The mechanism by which IGFBP-2 contributes to leptin-mediated weight loss is currently unknown. Numerous studies have shown that the growth hormone (GH) axis is perturbed in human obesity (Scacchi et al. 1999). In humans, plasma GH is inversely related to measures of adiposity, and obesity is associated with reduced plasma IGF-I, which is downstream of GH (Rudman et al. 1981) (Copeland et al. 1990). Treatment of patients with growth hormone results in increased lean mass and decreased adiposity (Rudman et al. 1990). In rodents, central leptin infusion stimulates spontaneous pulsatile GH secretion, as well as secretion in response to GHRH (Tannenbaum et al. 1998). In

addition, leptin reverses the starvation-induced fall in serum GH (LaPaglia et al. 1998). Thus, the leptin-specific induction of IGFBP-2 expression may be related to the normalization of the GH/IGF axis, and could thereby account for the role of IGFBP-2 in leptin-mediated weight loss.

Alternatively, the effects of IGFBP-2 may be independent of its role in the GH/IGF pathway. Recent reports have shown that IGFBP-s can also mediate actions independent of IGF, initiating signal transduction and acting as growth factors on their own (Mohan and Baylink 2002). IGFBP-s may also bind other circulating molecules, perhaps stabilizing them and increasing their half-life. IGFBP-4 can bind circulating leptin as part of a macromolecular complex (Sui and Wilson 2000). Since the IGFBP-s share a high degree of structural homology, IGFBP-2 could also bind leptin and stabilize it in the plasma, a possibility that is being explored. This would explain the higher leptin levels achieved in *ob/ob* mice capable of inducing IGFBP-2, and could ultimately account for the greater potency of leptin in these mice relative to IGFBP-$2^{-/-}$;*ob/ob* mice.

IGFBP-2 is part of a complex endocrine system with multiple levels of regulation. Its activity can be modulated by proteolysis, phosphorylation, and adherence to the cell membrane or extracellular matrix (Clemmons 1998). IGFBP-2 also has a specific pattern of temporal and tissue-type expression (Schneider et al. 2000). Finally, IGFBP-2 levels are altered in numerous disease states including obesity, diabetes, glioma, prostate cancer (Cohen et al. 1993; Nam et al. 1997; Frystyk et al. 1999; Zhang et al. 2002). Thus, further studies of animal models with tissue-specific deletion and overexpression of IGFBP-2 are necessary to dissect its pleiotropic physiological roles.

While the mechanism is not yet known, the data presented here indicate that IGFBP-2 induction plays an important role in leptin-mediated weight loss. While IGFBP-2 expression is completely suppressed in *ob/ob* and *db/db* mice, it is only partially suppressed in A^y and NZO mice, models of severe and moderate leptin resitance respectively. The presence of tonic IGFBP-2 expression in these mice may underlie their partial response to leptin therapy and suggests that co-administration of leptin and IGFBP-2 may augment their weight loss response. Leptin resistance appears to be a feature of the majority of human obesity, and obese humans also have suppressed levels of IGFBP-2 levels (Nam et al. 1997; Frystyk et al. 1999). These findings suggest that IGFBP-2 could be useful as a leptin adjuvant in the therapy of obesity and metabolic diseases.

Chapter 8: Conclusion

Summary of findings

The studies in this thesis explore the molecular basis for the metabolic effects of leptin. We specifically studied the effects of leptin on the liver, due to its prominent role in carbohydrate and lipid metabolism. Leptin-deficient *ob/ob* mice manifest a mixed form of hepatic insulin sensitivity and resistance, resulting in increased lipogenesis along with elevated gluconeogenesis, which contributes to both obesity and diabetes (Shimomura et al. 2000). In the absence of effective leptin action, triglyceride is deposited in peripheral sites such as liver, skeletal muscle, heart, and pancreatic islets. This build-up of lipid in non-adipose tissues causes lipotoxicity, which contributes to insulin resistance and organ dysfunction in the obese state (Unger 2002) (McGarry 1992; Shulman 2000). Leptin replacement in leptin-deficient (*ob/ob*) mice and humans leads to the specific depletion of lipid in adipose tissue, liver, and other tissues (Campfield et al. 1995; Halaas et al. 1995; Pelleymounter et al. 1995; Farooqi et al. 1999). The metabolic program that leptin elicits is not explained by its effects on food intake alone (Levin et al. 1996; Kamohara et al. 1997; Shimomura et al. 1999c). Leptin, with significantly greater potency than pair-feeding, rapidly reduces lipid in non-adipose tissues by decreasing triglyceride formation and increasing free fatty acid oxidation (Shimabukuro et al. 1997a). The goal of this thesis was to dissect the molecular basis for leptin's effects on hepatic metabolism in order to better understand the unique program of leptin-mediated weight loss.

As an entry point to this question, we first examined whether leptin's effects on liver were the result of direct action on heptocytes or secondary to action on the brain (Chapter 3). While data from intracerebroventricular administration of leptin suggested that it has direct effects on brain, the leptin receptor is broadly expressed, and leptin has also been shown to act directly to deplete triglycerides (Campfield et al. 1995; Ghilardi et al. 1996; Fei et al. 1997; Halaas et al. 1997; Shimabukuro et al. 1997a; Minokoshi et al. 2002). The relative importance of leptin's effects on brain vs. peripheral sites was untested as was the contribution of these actions to body weight homeostasis, deposition of lipid in peripheral sites, and neuroendocrine function. Therefore, we generated and studied mice with either neuronal ($ObR^{SynI}KO$) or hepatocyte-specific ($ObR^{Alb}KO$) deletions of ObR (Cohen et al. 2001). Among the $ObR^{SynI}KO$ mice, animals with the lowest levels of ObR exhibited an obese phenotype with elevated plasma levels of leptin, glucose, insulin, and corticosterone. These data demonstrated that leptin has direct effects on neurons and that a significant proportion, or perhaps the majority, of its weight reducing and neuroendocrine effects result from its actions on the brain. $ObR^{Alb}KO$ mice, on the other hand, weighed the same as controls with no alterations in body composition. Moreover, while *db/db* and $ObR^{SynI}KO$ mice had enlarged fatty livers, $ObR^{Alb}KO$ mice did not. While these findings do not exclude a functional role for the hepatic leptin receptor, they do indicate that the severe liver abnormalities of *ob/ob* and *db/db* mice are secondary to defective leptin signaling in the brain.

The demonstration that the brain is a critical site of leptin action suggested that leptin modulates a neuronal circuit regulating peripheral metabolism. The components of this neuronal circuit and the nature of the efferent signals from the central nervous system

that regulate metabolism in response to leptin are unknown. Since leptin elicits a metabolic response that cannot be explained by its effects on food intake alone, we hypothesized that leptin causes weight loss via a novel program, distinct from food restriction on both a physiological and molecular basis (Levin et al. 1996). This program was presumed to involve modulation of gene expression in metabolically active organs such as liver, adipose tissue, and muscle, associated with the depletion of peripheral lipid (Soukas et al. 2000). In order to more fully characterize this metabolic response, we examined leptin-specific gene expression in liver (Chapter 4). We identified a novel, distinct transcriptional signature characteristic of the unique metabolic program of weight loss induced by leptin. These data distinguish leptin treatment from food restriction at a molecular level, and may ultimately provide new insight into the nature of the leptin-stimulated efferent signals from the brain.

We also presumed that a subset of these leptin-regulated genes might play a functional role in the obese phenotype and the unique metabolic actions of leptin. To prioritize such genes for further analysis, we developed an algorithm to identify genes that were repressed during leptin-mediated weight loss (Chapter 5). Leptin was shown to specifically repress RNA levels and enzymatic activity of hepatic stearoyl-CoA desaturase-1 (SCD-1), which catalyzes the biosynthesis of monounsaturated fatty acids. Mice lacking SCD-1 were hypermetabolic and lean. *ob/ob* mice with mutations in SCD-1 (*ab*^{J}/*ab*^{J}; *ob/ob*) were significantly less obese than *ob/ob* controls and demonstrated markedly increased energy expenditure. *ob/ob* mice with mutations in SCD-1 had histologically normal livers with significantly reduced triglyceride storage and VLDL production. These data suggested that down-regulation of SCD-1 is an important

component of leptin's metabolic actions and that inhibition of this enzyme may be beneficial for the treatment of obesity and other metabolic diseases.

SCD-1 is required for normal rates of hepatic triglyceride and cholesterol ester biosynthesis and *ob/ob* mice lacking SCD-1 (*ab^J/ab^J; ob/ob*) displayed a dramatic correction in the hepatic steatosis typical of *ob/ob* mice (Miyazaki et al. 2000). Therefore, we examined whether SCD-1 plays a more general role in fatty liver disease (Chapter 6). Hepatic steatosis is the earliest sign of both alcoholic and nonalcoholic fatty liver disease. SCD-1 expression is elevated in livers of mice with alcoholic fatty liver and in mice with nonalcoholic fatty liver disease (NFLD) secondary to lipodystrophy. Mice lacking SCD-1 (*ab^J/ab^J*) were resistant to the development of alcoholic steatosis, while littermate controls accumulated significant amounts of liver triglyceride. In addition, lipodystrophic mice lacking SCD-1 (*ab^J/ab^J;aP2-SREBP-1c tg*) showed a marked correction in the NFLD associated with this condition. Millions of people worldwide are affected by alcoholic fatty liver disease or NFLD and no effective treatment is currently available. Our findings suggest that targeted inhibition of SCD-1 may be therapeutically useful for these disorders.

The important role demonstrated for SCD-1 suggested that other leptin-regulated genes might also be functionally involved in leptin's unique metabolic effects. While *ob/ob* mice lacking SCD-1 showed a marked correction of obesity and other components of the obese phenotype, the absence of this gene alone did not completely eliminate the abnormalities of *ob/ob* mice. We therefore assumed that other alterations in gene expression, typical of *ob/ob* mice, persisted and contributed to the residual phenotype in these mice. Using a computational approach, we identified a group of genes that were

reduced in *ob/ob* liver and specifically induced by leptin. We reasoned that the induction of some of these genes might be required for leptin's metabolic effects. Insulin-like growth factor binding protein-2 (IGFBP-2) expression was uniquely induced by leptin administration and not by pair-feeding. *ob/ob* mice lacking IGFBP-2 (*IGFBP-2*$^{-/-}$*;ob/ob*) showed markedly attenuated weight loss following leptin treatment.

Directions for future research

The findings presented here have generated a number of questions which remain to be answered. With regard to leptin's central vs. peripheral action, more detailed studies using metabolic tracers may yet uncover a role for the hepatic leptin receptor in modulating lipid metabolism. Furthermore, hepatic ObR may only be functionally relevant in altered physiological states such as genetic or dietary obesity or marked hypo- or hyperleptinemia, possibilities that are currently being evaluated. However, the enlarged fatty liver in ObR^{SynI}KO mice and the absence of steatosis in ObR^{Alb}KO mice, clearly indicates that defective central leptin action is responsible for the liver pathology of *ob/ob* and *db/db* mice. Therefore, it will be important to define the neuronal circuit and to determine the nature of the efferent signal from the CNS that modulates the metabolic effects of leptin in liver. Given that most obese humans are leptin resistant and that likely sites of resistance are in the transport across the blood-brain-barrier and downstream CNS pathways, identifying the efferent signal(s) emanating from the brain in response to leptin could have great therapeutic benefits (Maffei et al. 1995b; Caro et al. 1996; Considine et al. 1996).

The efferent signal is part of a homeostatic pathway, in which elevated leptin levels signal nutritional abundance to the brain, which then activates thermogenesis to increase energy expenditure and maintain stable body weight (Lowell and Spiegelman 2000). The CNS efferent is likely to involve the autonomic system and/or the hypothalamic pituitary axis. The presence of obesity in mice lacking all three β–adrenergic receptors (β-less mice) indicates that the sympathetic nervous system is a critical component of the CNS efferent pathway activated by leptin (Bachman et al. 2002). Testing the response of these mice to leptin should allow the contribution of this limb of the CNS efferent pathway enacted by leptin to be evaluated.

The leptin-specific programs of liver gene expression identified in Chapter 4 may be valuable for further dissecting the CNS efferent pathways activated by leptin. Regardless of their functional role, these patterns of expression can be used as a molecular marker of leptin action. In addition to treating β-less mice with leptin, these mice can be also be bred on to the *ob/ob* background and treated with leptin (Bachman et al. 2002). Comparison of liver gene expression between these mice (β-less; *ob/ob*) and *ob/ob* mice might show that certain clusters of genes with leptin-specific patterns of expression in *ob/ob* liver were no longer modulated in a leptin-dependent manner. These clusters would likely contain genes that are regulated by leptin-dependent sympathetic activation. In theory, the role of any candidate efferent signal could be similarly tested in genetic models or determined by treating *ob/ob* mice with an agonist or antagonist of that pathway and monitoring leptin-specific gene expression.

Further understanding of the transcriptional regulation of leptin-specific patterns of gene expression can also be used to trace backwards to identify the efferent signal

emanating from the CNS. If a leptin-regulated transcription factor were identified, the next goal would be to determine the mechanism by which leptin modulates this factor. Such studies could elucidate post-translational modifications, signal transduction pathways, and even extracellular signals that are stimulated or repressed by leptin. It should be noted that in this discussion, the term *leptin-regulated* is used rather loosely. The transcriptional machinery regulating metabolism is highly complex and could be modulated by a multitude of factors. In addition, leptin may only regulate this pathway secondary to its effects on other hormones, such as insulin. In any case, the identification of such a factor would help elucidate the mechanism for leptin's novel metabolic actions.

A number of transcription factors regulate metabolic gene expression, and whether or not these factors are regulated by leptin remains untested. In addition, clusters of gene expression can also be used to computationally study transcriptional regulation. With the completion of the human and murine genomes, in prinicipal, the transcriptional start sites and upstream promoter sequence of every clustered gene can be obtained. Computational methods can then query these sequences and determine whether either known or novel promoter motifs are enriched in particular clusters. This could lead to the identification of a "leptin-regulated element" which could then be used to biochemically purify the transcription factor which binds it. Such an approach has been successfully applied to identify a novel circadian regulated transcription factor in mice (Ueda et al. 2002).

The more detailed characterization of leptin-specific regulation of SCD-1 and IGFBP-2 suggests that these genes may be useful for studying the transcriptional control of leptin-repressed and leptin-induced gene expression, respectively. Of interest, both of

the RNA levels of both of these genes also appear to regulated by leptin administration in wild-type mice (data not shown). As discussed in Chapter 4, very few genes were specifically modulated by leptin in wild-type liver, and these genes are more likely to be specifically modulated by quantitative changes in circulating leptin levels. Prior to beginning these studies, nuclear run-on assays will be performed to test whether regulation of these genes is at the level of transcription and not at the level of RNA stability. If regulation does prove to be transcriptional, constructs fusing different portions of the promoter to a CAT or luciferase indicator will be made. The human SCD-1 promoter has been cloned and a number of different luciferase-promoter constructs have already been studied *in vitro* (Bene et al. 2001). Generation of transgenic mice harboring these constructs will allow *in vivo* analysis of leptin-dependent regulation of gene expression. These studies will permit the delineation of promoter regions required for leptin-dependent gene expression. The identification of these sequences will be a useful reagent for studying the signal transduction pathways and transcription factors that regulate these genes.

The data presented suggests one possible mechanism by which leptin regulates SCD-1 expression. The transcriptional modulation of SCD-1 is complex, with a number of factors known to stimulate and repress transcription (Ntambi 1995). Polyunsaturated fatty acids (PUFAs) repress SCD-1 transcription through a defined promoter element, independent of their reported ability to inhibit SREBP cleavage and nuclear translocation (Yahagi et al. 1999; Bene et al. 2001; Ntambi and Bene 2001). Fatty acid composition of *ob/ob* livers demonstrated that leptin-specifically increased the levels of PUFAs, which

are otherwise repressed in *ob/ob* liver (Table 5.2). These increased PUFA levels could, in turn, account for the leptin-dependent suppression of SCD-1 RNA levels.

As well as being used as molecular markers of leptin's effects, SCD-1 and IGFBP-2 have functional roles in metabolism and energy homeostasis. With regard to SCD-1, future studies on inbred *ab^J/ab^J;ob/ob* mice will determine whether other metabolic abnormalities of the obese phenotype such as insulin resistance and diabetes are corrected in the absence of SCD-1. Neuroendocrine, thermoregulatory, immune, and reproductive function will also be examined. In addition, the phenotype of these mice will be characterized at an older age to determine whether there are any undesirable side effects of eliminating SCD-1.

Understanding the mechanism underlying the effects of SCD-1 deficiency will also be a focus of future studies. Figure 5.5a proposed one possible mechanism by which the absence of SCD-1 could lead to increased fatty acid oxidation and increased energy expenditure. While Figure 5.5b presented preliminary data illustrating that *ab^J/ab^J* and *ab^J/ab^J;ob/ob* mice have elevated saturated fatty acyl CoAs, other elements of this hypothesis require further testing. Measurement of malonyl CoA levels is necessary to confirm whether these saturated fatty acyl CoAs might inhibit ACC. In addition, *ab^J/ab^J* and *ab^J/ab^J;ob/ob* mice will be treated with etomoxir, an inhibitor of CPT-1, to determine whether the increased oxygen consumption is blunted when fatty acid translocation into the mitochondria and subsequent oxidation is blocked (Esser et al. 1993). Finally, measurement of tissue-specific fatty acid oxidation in these mice is necessary to determine whether increased fatty acid oxidation, or another mechanism altogether, is

responsible for the increased energy expenditure and resistance to obesity in mice lacking SCD-1.

These same studies will also be performed in alcohol fed ab^J/ab^J mice and in ab^J/ab^J;*aP2-SREBP-1c tg* mice to see if similar mechanisms are operative in their apparent resistance to fatty liver disease. In addition, ab^J/ab^J mice have been bred to other mouse models of fatty liver disease including mice with transgenic overexpression of nuclear sterol regulatory element-binding protein-1a (*PEPCK-SREBP-1a tg*) and A^y mice (Yen et al. 1976; Shimano et al. 1996). In addition to assessing resistance to hepatic steatosis, all of these models will be followed over time to see if they are similarly resistant to fibrosis and other advanced pathology of fatty liver disease. Furthermore, mice lacking SCD-1 will be treated with carbon tetrachloride and thioacetamide, inducers of hepatic fibrosis, to determine whether their resistance to hepatic lipid accumulation renders them resistant to more severe liver pathology as well (Friedman 2000b).

Future studies will also explore whether SCD-1 and SCD-2 expression, activity, and enzymatic products, are modulated by leptin in tissues other than liver. While the effects of SCD-1 deficiency described are attributable to an increased rate of energy expenditure, the full complement of tissues contributing to this increased energy expenditure are as yet unknown. *ob/ob* mice were followed over a time course of leptin administration, saline with pair-feeding, and saline with free-feeding (identical to the experiment described in Chapter 4) and liver, fast and slow twitch skeletal muscle, white and brown adipose tissue, heart, hypothalamus, and other tissues were harvested for this analysis. Leptin was recently shown to activate fatty acid oxidation in heart, independent

of α2 AMP-kinase activation, suggesting that another pathway, potentially involving regulation of SCD-1 could be relevant (Atkinson et al. 2002).

In addition, analysis of SCD-1 regulation in hypothalamus and other brain regions will be important to determine whether there is a direct role for SCD-1 and its products in the neuronal pathways controlling feeding. Central administration of C75, an inhibitor of fatty acid synthase, has indicated that alterations in hypothalamic malonyl CoA levels are associated with reduced levels of NPY, leading to reduced food intake and weight loss (Loftus et al. 2000). *ab^J/ab^J;ob/ob* mice are hyperphagic relative to *ob/ob* mice, perhaps secondary to increased energy expenditure or possibly due to a compensatory increase in SCD-2 expression and/or activity in the brain. Understanding the mechanism for the hyperphagia in *ab^J/ab^J;ob/ob* mice and its consequent blunting, could lead to an even greater correction of the obese phenotype in these animals. To explore whether there is a compensatory increase in other SCD isoforms in mice lacking SCD-1, SCD-2 and SCD-3 expression, SCD activity, and enzymatic products will also be measured in multiple tissues from *ab^J/ab^J;ob/ob* mice. Data generated thus far indicates that there is no compensation by other SCD isoforms in the liver, as SCD activity in *ab^J/ab^J* and *ab^J/ab^J;ob/ob* mice could not be detected. Similar studies are underway in other tissues, including the hypothalamus.

Generation of mice with tissue-specific overexpression of different SCD isoforms is another means to dissect the role of this molecule in individual tissues. Constructs directing hepatocyte-specific overexpression of SCD-1 and SCD-2 are currently being generated. The demonstration of markedly elevated hepatic SCD-1 expression and activity in the hyperlipidemic, *hyplip* mutant mouse suggests that hypertriglyceridemia

and hypercholesterolemia could be one consequence of hepatic overexpression of SCD-1 (Attie et al. 2002; Bodnar et al. 2002). Based on the analysis of leptin-specific SCD regulation in other tissues, described above, transgenic overexpression could also be targeted to other relevant sites.

The data demonstrating a role for IGFBP-2 in leptin-mediated weight loss are more preliminary than that for SCD-1, and therefore require further experimentation. The data presented here suggest that *IGFBP-2*$^{-/-}$*;ob/ob* mice have a more severe phenotype than *ob/ob* controls. A complete analysis of the metabolic and other alterations characteristic of the obese phenotype need to be performed in inbred *IGFBP-2*$^{-/-}$*;ob/ob* mice. As IGFBP-2 is widely expressed, further studies are necessary to determine whether leptin-dependent induction of IGFBP-2 RNA is unique to liver or whether other tissues are also involved. To further dissect the effects of liver-specific induction of IGFBP-2, transgenic mice overexpressing IGFBP-2 exclusively in heptocytes and an adenovirus producing IGFBP-2 are currently being generated.

The mechanism for the apparent leptin resistance in *IGFBP-2*$^{-/-}$*;ob/ob* mice and the role for IGFBP-2 induction in leptin-mediated weight loss are unknown. Administration of recombinant IGFBP-2 and infection of mice with an IGFBP-2 adenovirus (currently being made) will allow for further study of the effects of increased levels of this molecule. The findings presented here could be due to an indispensable role for IGFBP-2 in the induction of the GH / IGF axis or could be due to an IGF independent role of IGFBP-2. Future experiments will be necessary to assay the effects of leptin on other components of the GH / IGF axis in *ob/ob* mice with and without IGFBP-2. One possible IGF-independent role for IGFBP-2 may be to stabilize circulating leptin, thereby

increasing its half-life and potentiating its actions. This possibility can be tested by comparing the leptin dose-response curves of *ob/ob* and *IGFBP-2*$^{-/-}$*;ob/ob* mice. Alternatively, this hypothesis could be explored by treating mice with different doses of recombinant IGFBP-2 and leptin or by co-infecting mice with different titers of IGFBP-2 and leptin adenoviruses. If the observed effects of IGFBP-2 are, in fact, independent of IGF, then the role for this molecule may be further dissected by analyzing gene expression in mice following IGFBP-2 administration or adenoviral infection. Perhaps, IGFBP-2 initiates a signaling pathway that represses anabolic gene expression and / or induces catabolic gene expression.

A common biochemical pathway determining nutrient partitioning

Analysis of leptin-specific gene expression in the liver has identified two molecules, SCD-1 and IGFBP-2 with functions in energy metabolism. SCD-1, and perhaps IGFBP-2 as well, is involved in determining whether calories are partitioned into fat or burned. Based on the findings in *ab*^{J}*/ab*^{J}*;ob/ob* mice, SCD-1 joins a list of other genes, that when either deleted or overexpressed confer leanness. Thus far, approximately 50 such genes have been described and the mechanisms underlying their effects have been divided into eight major categories: (1) peripherally driven increased energy expenditure in muscle, (2) peripherally driven increased energy expenditure via adipose tisse, (3) increased energy expenditure via multiple or unknown tissues, (4) CNS-mediated, (5) peripherally decreased energy availability, (6) abnormal adipocyte biochemistry, (7) white fat ablation, and (8) unknown (Reitman 2002). SCD-1 could be

placed in the third grouping, pending elucidation of the tissue(s) driving increased energy expenditure in mice lacking this gene.

Further analysis of genetic alterations conferring leanness indicates that a number of other molecules in the same biochemical pathway as SCD-1 are also involved in energy partitioning (Figure 8.1). Deletion of acetyl-CoA carboxylase 2 produces a lean phenotype, secondary to decreased malonyl CoA and increased fatty acid oxidation (Abu-Elheiga et al. 2001). Inhibition of fatty acid synthase by the compound C75 also leads to reduced adiposity (Loftus et al. 2000). This molecule was originally thought to act centrally to decrease food intake, but has been subsequently found to increase energy expenditure and peripheral fatty acid oxidation (Thupari et al. 2002). The finding of increased fat oxidation in the setting of elevated malonyl CoA was paradoxical. However, studies have suggested that C75 competes with malonyl CoA, thereby disinhibiting its actions on CPT-1 (Thupari et al. 2002). As described in this work, in the absence of SCD-1, animals demonstrate resistance to obesity and increased energy expenditure, perhaps also due to effects on malonyl CoA and fatty acid oxidation (Chapter 5)(Cohen et al. 2002). Furthermore, mice with a targeted deletion of acyl CoA:diacylglycerol transferase (DGAT), which catalyzes the final step in triglyceride biosynthesis, have increased energy expenditure and are protected from obesity (Smith et al. 2000). DGAT null mice also have sebaceous gland defects very similar to those in *asebia* mice (Chen et al. 2002). Interestingly, a promoter polymorphism in this gene leading to decreased expression is associated with reduced BMI in Turkish women (Ludwig et al. 2002). Modulation of the above gene products may prevent obesity by a similar mechanism to

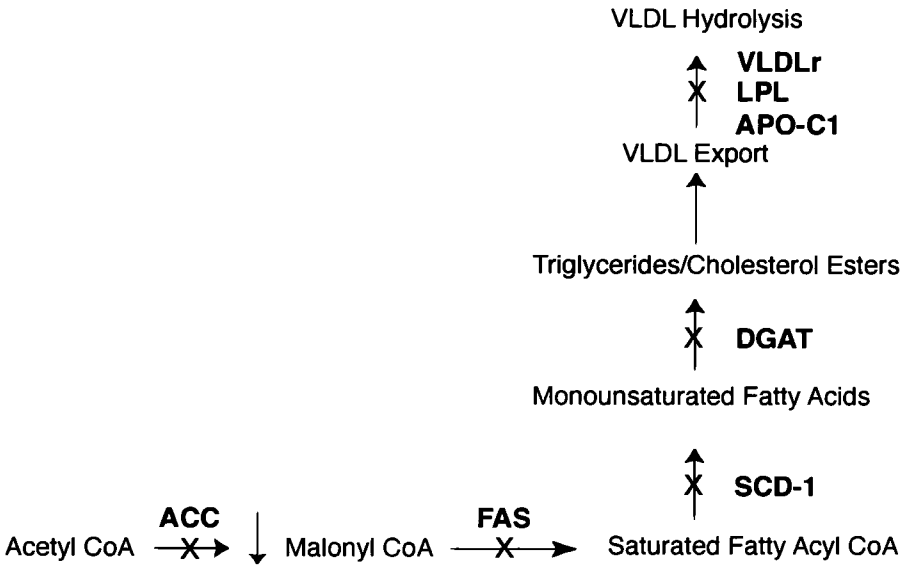

Figure 8.1 SCD-1 is part of a biochemical pathway regulating energy partitioning and adiposity. As described in the text, modulation of the gene targets in bold print affect body weight. In all cases (except for Apo-C1, which exerts its effects when over-expressed), inhibition of the gene target protects mice from obesity. ACC: acetyl-CoA carboxylase 2, FAS: fatty acid synthase, SCD-1: stearoyl CoA desaturase-1, DGAT: acyl CoA:diacylglycerol transferase, VLDLr: very low density lipoprotein receptor, LPL: lipoprotein lipase, APO-C1: apolipoprotein C1.

that proposed for SCD-1 in Figure 5.5a. Therefore, it will be important to determine the common and unique features of each of these models.

In the liver, the triglycerides formed through this pathway can be packaged in VLDL and exported to adipose tissue and other sites. A number of genetic alterations, inhibiting hydrolysis and consequently delivery of VLDL triglyceride, also lead to reduced adiposity (Figure 8.1). Mice lacking the VLDL receptor are lean, and breeding of this mutation on to the *ob/ob* background leads to a marked correction in obesity (Frykman et al. 1995; Goudriann et al. 2001). In addition, *ob/ob* mice lacking adipose tissue lipoprotein lipase (LPL), an enzyme required for triglyceride hydrolysis, also show diminished weight and fat mass (Weinstock et al. 1997). Finally, *ob/ob* mice overexpressing of apolipoprotein C1 (apo-C1), which has been proposed to inhibit hydrolysis of VLDL triglyceride, demonstrate a nearly total correction in obesity (Jong et al. 2001).

Analysis of other leptin-regulated genes in Table 5.1 and Table 7.1 may identify other molecules that can protect from obesity, operating through the aforementioned pathway or through an entirely different mechanism. One such gene may already exist in corticosteroid binding globulin (CBG), which is reduced in *ob/ob* liver and specifically induced by leptin (Figure 4.1, Table 7.1). Obese Zucker rats have reduced levels of CBG, which binds corticosterone in the circulation and limits its tissue availability (Grasa et al. 2001). Increased tissue corticosterone levels lead to visceral obesity and metabolic defects in mice (Masuzaki et al. 2001). In addition, an obese human proband has been identified with total genetic deficiency in CBG (Roitman et al. 1984).

The continued study of SCD-1, IGFBP-2, and other leptin-regulated genes in liver should help further elucidate the mechanism for leptin's effects on metabolism and the means by which these genes modulate body weight. In addition, microarray analysis of liver gene expression in *ab^J/ab^J;ob/ob* mice should also be of value in determining which expression alterations in *ob/ob* mice are corrected and which remain dysregulated. Functional study of both groups of genes may identify other molecules involved in energy homeostasis.

Conclusion

Overweight and obesity are a pressing public health problem. With increased prosperity and development, obesity is gradually replacing starvation and malnutrition as the world's most common metabolic disease. The discovery of leptin confirmed that body weight and appetite are under molecular control. Identification of other molecules involved in leptin's endocrine loop has demonstrated other control points for body weight, some of which may be amenable to pharmacologic manipulation. The polygenic nature of obesity and the complex interplay of genes and environment in this disorder make it unlikely that a single gene product or drug will be found to cure all human obesity. However, targeted manipulation of numerous points in the molecular machinery regulating energy homeostasis may alleviate this condition. Therefore, the complete elucidation of leptin's circuit and the identification of the full complement of molecules involved in its unique metabolic effects may provide novel therapeutic targets. The use of oligonucleotide microarrays to identify such genes coupled with functional analysis of these molecules should contribute to this effort.

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