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The effects of leptin and thyroid hormone on metabolism and lipid trafficking in the liver and adipose tissue of ob/ob mice

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THE EFFECTS OF LEPTIN AND THYROID HORMONE ON METABOLISM AND LIPID TRAFFICKING IN THE LIVER AND ADIPOSE TISSUE OF *OB/OB* MICE

A Thesis

Presented to the

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California State University,

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In Partial Fulfillment

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ABSTRACT

Altered metabolism can occur when the metabolic hormones, leptin and thyroid hormone become unresponsive to blood nutrient levels. Leptin is secreted from adipose tissue in response to food intake, resulting in appetite suppression and increased metabolism. Thyroid hormone (T_3) regulates genes involved in energy utilization, lipolysis, and lipogenesis, and stimulates metabolism.

Obese mice (*ob/ob*) are leptin deficient and are widely used to study the effects of leptin on metabolism. In this study, twenty nine week old C57BL/6J *ob/ob* mice were injected for seven days with vehicle, leptin, iopanoic acid (IOP, to reduce T_3 concentration), or a combination of leptin and IOP (n=5/group). Mice were analyzed for changes in mass-specific oxygen consumption, respiratory quotient, body weight, and food intake over the treatment period. Hepatic lipid content and lipid droplet size, and adipocyte area were determined post mortem.

Leptin decreased liver lipid content and lipid droplet size, and increased omental adipocyte area, suggesting leptin has the ability to reduce excess lipid storage in the liver by shuttling lipid to adipocytes. The IOP treated mice were not different from the vehicle treated mice with

regards to adipocyte area and liver lipid content. The leptin/IOP treated mice had increased hepatic lipid content and lipid droplet size, and the smallest adipocytes. This suggests that reduced thyroid hormone is the limiting factor regarding metabolizing and exportation of hepatic lipids, and suggests a possible link between thyroid hormone and leptin in regulating lipid metabolism.

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This project could not have been completed without the guidance of many individuals. My undergraduate and graduate advisor, Dr. Richard Fehn, whom passed away during my first year, taught me all the necessary techniques to conduct my experiments and provided me with much needed background knowledge on the topic. Without him, I would have never felt so much passion for a field or learned the valuable life lessons he taught me. I cannot begin to describe how much he means to me. He is deeply missed. I would also like to thank Dr. Colleen Talbot, who stepped up to become my major advisor when I needed someone. Without her continued help and support, this thesis would not have been completed. I thank my committee members Dr. James Ferrari, Dr. Mike Chao, and Dr. Kevin Middleton for their feedback provided on the statistical analyses and end product of this thesis. Dr. David Polcyn, as well as those mentioned, provided much needed emotional support during the difficult times. Without the support of the entire biology faculty (I can't name everyone), I would have lost my way and not finished this thesis. There are no words that can describe the gratefulness I feel for the amazing faculty here. I thank Cindy Chrisler for her help with the care of the

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CSUSB is and will always be my home. The biology department faculty and students are a part of my life forever. But it is now time for me to go on with life, taking my principles learned here with me.

"At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us." -Albert Schweitzer

"It was now too late and too far to go back and I went on. And the mists had all solemnly risen now, and the world lay spread before me" -Charles Dickens

"To Dr. Richard Fehn, in loving memory"

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CHAPTER ONE

LITERATURE REVIEW OF ENERGY HOMEOSTASIS

Background

Obesity has become an increasingly serious problem in the Western hemisphere and can cause serious health problems, primarily cardiovascular disease. The cellular mechanisms underlying the development of obesity have been obscure, but in 1994 the discovery of the hormone leptin changed the way obesity and diabetes research was conducted and provided a new way to approach the study of the metabolic syndrome (Zhang et al. 1994). Obesity may result from improper eating, leading to an increase in fat stores, which leads to an altered metabolism. Altered metabolism occurs when cells that produce hormones involved in nutrient trafficking become unresponsive to blood nutrient levels, resulting in uncontrolled appetite and improper storage of nutrients. It is important to study how the key metabolic hormones, insulin, leptin, and thyroid hormone, alter metabolic pathways and lipid and glucose trafficking in order to find possible therapies for treatment of obesity and its complications.

Energy Homeostasis

Energy homeostasis is the regulation of body weight by the brain and peripheral tissues to maintain a stable, internal environment in the presence of a dynamic external environment. This is dependent upon the balance between food intake as well as anabolic and catabolic pathways, or the building up and breaking down of molecules, respectively. Particularly, signals from peripheral tissues are transmitted to the brain when a change in body energy reserves are "sensed" due to alterations in energy expenditure, nutrient partitioning, and energy intake.

Maintenance of energy homeostasis is a complex process that is mediated by multiple endocrine and neurocrine feedback loops to and from the central nervous system (CNS) and involves cross-talk among many tissues, including the brain, liver, skeletal muscle, stomach, pancreas, and adipose tissue. The sympathetic nervous system (SNS) of the CNS regulates whole body metabolism by its actions on neurons of the hypothalamus. These neurons secrete neurohormones (releasing hormones) that trigger the secretion of hormones from the anterior pituitary gland. In turn, the pituitary gland links the nervous and endocrine systems. The anterior pituitary hormones bind to specific

cellular receptors, eliciting their respective responses, which can then feedback to the SNS to turn down their secretion.

The SNS is important in regulating appetite. Feeding is regulated by signals from the arcuate nucleus located within the hypothalamus (Elmquist et al. 1998b). Neuropeptide Y (NPY) and agouti-related peptide (AgRP) stimulate appetite while proopiomelanocortin (POMC) and melanocortin stimulating hormone (α-MSH) induce a decrease in appetite (figure 1). NPY is also known to inhibit thermogenesis (Billington et al. 1991; Dryden and Williams 1996). Decreased thermogenesis reduces oxygen consumption, allowing nutrients (glucose and lipids) to be stored instead of being utilized to support oxygen consumption. A rise in stored nutrients reduces appetite stimulation so that overeating does not occur.

Neurons of the hypothalamus are stimulated by the hormones ghrelin, insulin, and leptin, which in turn are regulated by circulating glucose and free fatty acid levels and intracellular glycogen and triacylglycerol (TG) stores (Houseknecht 1998). While ghrelin stimulates appetite through AgRP and NPY, insulin and leptin cause appetite inhibition through inhibition of the same neurons and

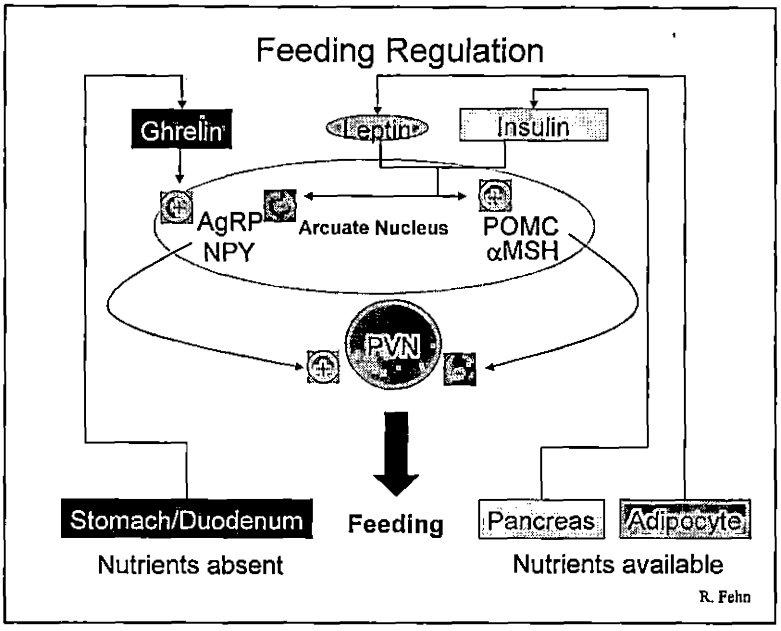


Figure 1. Hormonal Control of Feeding Regulation. Feeding regulation is controlled by hormones released from the stomach, pancreas, and adipose tissue. When nutrients are available, insulin and leptin are secreted from the pancreas and adipocytes, respectively, to inhibit AgRP and NPY while simultaneously stimulating POMC and αMSH. This inhibition and stimulation leads to an inhibition of feeding on the PVN. When nutrients are absent, ghrelin is released from the stomach/duodenum and stimulates AgRP and NPY, which leads to a stimulation of feeding on the PVN. AgRP= agouti-related peptide; NPY= Neuropeptide Y; POMC= proopiomelanocortin; αMSH= melanocortin stimulating hormone; PVN= paraventricular nucleus

stimulation of POMC and α-MSH (figure 1). (Due to the focus of this thesis, ghrelin will not be discussed. For further information see review by Lazarczyk et al. 2003.) Insulin is secreted from pancreatic β cells when plasma glucose levels rise after a meal and through its actions on the

hypothalamus, it stimulates glucose and free fatty acid uptake into peripheral tissues. Glucose uptake in skeletal muscle and adipose tissue is directly dependent on insulin. However, in the liver, insulin indirectly facilitates diffusion of glucose into hepatocytes, but some glucose can still diffuse into hepatocytes even in the absence of insulin. When glucose enters hepatocytes, it can immediately enter glycolysis, increasing the production of cellular energy, or be converted to its storage form, glycogen. If insulin is oversecreted due to chronic elevated plasma glucose (e.g. from overeating), its signal on cells will be ignored, referred to as accommodation or insulin resistance. Insulin resistance will decrease glucose uptake and lead to hyperglycemia (Houseknecht et al. 1998a).

While insulin elicits its responses over the short term (hours), leptin can elicit the same responses over the longer term (days). Leptin is a hormone of the cytokine family and is secreted by adipose cells in response to energy intake (Tartaglia et al. 1995). In addition to insulin-like effects, leptin can also control feeding behavior (Houseknecht et al. 1998b). For instance, when adipocytes increase in size due to a post-prandial (after

meal) increase in stored nutrients, leptin is secreted from adipocytes to inhibit appetite. Lack of appetite inhibition, i.e. low leptin levels, can lead to overeating. There is an importance in understanding the changes in glucose and lipid metabolism in response to nutritional status in obese individuals, who may be leptin deficient (Houseknecht et al. 1998b).

Leptin gene expression is regulated by the size of adipocytes as well as insulin and glucocorticoids (Caro et al. 1996). There is a positive correlation between adipose tissue mass and fat cell size to the quantity of leptin secreted (Houseknecht et al. 1998a; Caro et al. 1996). Adipose cells sense excess fat storage and stimulate the release of leptin, which then leads to an increase in total body energy expenditure, decreased food intake, and normalization of body weight in obese mice within 2 weeks or less (Halaas et al. 1995; Maffei et al. 1995). Leptin acts either directly, binding to receptors on its target cells (e.g. liver), or indirectly by binding to leptin receptors in the hypothalamus because leptin is able to penetrate the blood-brain barrier. Leptin that targets the hypothalamus then stimulates the release of neurohormones, such as thyroid stimulating hormone and growth hormone

(Houseknecht et al. 1998b), from the pituitary to induce their respective cellular responses (Elmquist et al. 1998b; Houseknecht et al. 1998b; Fehn et al. 1999). Pelleymounter et al. (1995) showed that leptin administration to leptin deficient obese mice (*ob/ob*, refer to section "the *ob/ob* mouse model", p. 23) stimulated an increase in basal metabolic rate and thermogenesis, leading to a reduction in body weight. The administration of leptin to *ob/ob* mice decreases food intake and restores neuroendocrine functions that are lost as a result of leptin deficiency. These include infertility derived from decreased hypothalamic leutinizing hormone secretion in *ob/ob* mice, among other reproductive abnormalities (Halaas et al. 1995; Pelleymounter et al. 1995; Campfield et al. 1995; Chehab et al. 1996). Furthermore, Fehn et al. (1999) showed that leptin administration also increased thyroid hormone concentrations in *ob/ob* mice due to enhanced thyroid follicular cell activity leading to enlarged colloids. Reno and Fehn (2007) also showed that SNS-ablated *ob/ob* mice and hyperthyroid *ob/ob* mice were rescued from hepatic steatosis (fatty liver) with administration of leptin. This result shows that leptin can elicit physiological responses independently of the SNS and in the presence of excess

thyroid hormone, supporting the importance of leptin in maintaining energy homeostasis. However, the extent of these responses has only been studied at a histological level and needs to be examined further by molecular analyses. Additionally, when the SNS is activated by leptin, lipolysis in white adipose tissue is stimulated (Siegrist-Kaiser et al. 1997; Fruhbeck et al. 1997), releasing free fatty acids for use in metabolism.

The pathologies associated with obesity and diabetes, among others, are often linked with either a complete absence of leptin or the brain's inability to respond to leptin (i.e. leptin resistance) because without leptin's actions, there is improper storage and release of glucose and fatty acids, primarily in adipocytes and hepatocytes (Halaas et al. 1995; Pelleymounter et al. 1995; Campfield et al. 1995; Chehab et al. 1996).

Even though leptin can affect energy metabolism through the hypothalamus (i.e. CNS) and/or through receptors located directly on peripheral tissues (Elmquist et al. 1998; Houseknecht et al. 1998b; Fehn et al. 1999; Fei et al. 1997; Elmquist et al. 1998a; Mercer et al. 1996; Schwartz et al. 1996), research has primarily focused on its CNS effects (Tartaglia et al. 1995; Caro et al. 1996;

Halaas et al. 1995; Pelleymounter et al. 1995). Recently, this focus has shifted to trying to understand the mechanisms of direct leptin stimulation of peripheral target cells. The cellular responses to leptin are dependent on the concentrations of circulating glucose, free fatty acids, and other hormones such as insulin (Houseknecht et al. 1998a; Stephens and Caro 1998). The liver is one of the main regulators of macronutrient metabolism, controlling serum free fatty acid and glucose levels in response to serum leptin concentrations. Serum glucose levels affect the activity of key enzymes involved in metabolic processes, such as glycolysis and β-oxidation. After sufficient quantities of glucose are taken up into tissues (primarily liver) to meet energy requirements, glucose is converted into its storage form, glycogen, in order to prevent excess secretion of glucose back into the blood. During the post-prandial period, a rise in serum glucose levels causes pancreatic β cells to secrete insulin (figure 2), which activates glycogen synthetase and leads to an increase in hepatic glycogen storage. In a fasting state, pancreatic α cells respond to the serum glucose deficiency and secrete glucagon, which together with epinephrine activates phosphorylase in the liver to convert

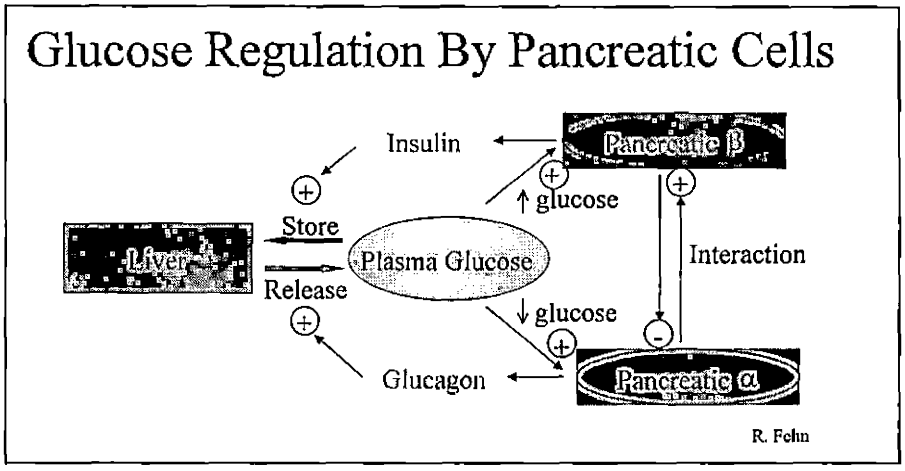


Figure 2. Glucose Regulation by Pancreatic α and β Cells. Increases in plasma glucose are sensed by pancreatic β cells, which secrete insulin, signaling the liver to store glucose. High levels of plasma glucose also inhibit glucagon secretion from pancreatic α cells. Plasma glucose deficiency is sensed by pancreatic α cells, leading to glucagon secretion and the release of glucose from the liver. Pancreatic α cells also stimulate insulin secretion from pancreatic β cells, preventing hyperglycemia due to hepatic glucose release.

glycogen into glucose. This glucose is released from the liver into the blood to be used for cellular energy by peripheral tissues via glycolysis. Since glycolysis produces acetyl-CoA, which is required to produce fatty acids, the concentration of glucose or glycogen present in the liver can indirectly control the rate of fatty acid formation, which can affect the amount of fatty acid stored as TG. For example, if hepatic glucose concentrations increase to support an increase in glycolysis, then more

fatty acids can be built, causing the excess fatty acids to be stored as TG (Gibbons et al. 2000).

In addition to stimulating the conversion of glycogen to glucose, glucagon can also stimulate lipolysis in adipose tissues, which decreases the size of adipocytes and provides free fatty acids to potentially be utilized in hepatic glucogenesis. Furthermore, glucagon stimulates the activity of enzymes that lead to the β-oxidation of fatty acids. Abnormalities associated with increased serum glucagon can cause the release of excess glucose and fatty acids into the blood, which then leads to altered insulin sensitivity. If insulin sensitivity is decreased, then the storage and release of glucose will be decreased and that of fatty acids increased. This reduced glucose uptake into adipose tissue and skeletal muscle due to insulin resistance can lead to hyperglycemia, hyperlipidemia, and/or excess TG storage in the liver. The overproduction of both hepatic glucose and fatty acids can occur as a result of chronic overeating. This leads to a mistaken sensation of low plasma glucose and fatty acids by the pancreas due to altered glucose and lipid trafficking. Overproduction of glucose and fatty acids leads to hypersecretion of these molecules into the blood. This

causes a rise in serum glucose and free fatty acid levels and stimulates the release of more insulin from pancreatic β cells, which will exacerbate peripheral insulin resistance (McGarry 1992), a key factor in the development of obesity and type II diabetes. Once insulin resistance occurs, there is a continual sensation of excess macronutrients in the blood, and this condition will cause excessive storage of the glucose and fatty acids, not only in adipocytes, but in hepatocytes as well. Therefore, the control of glucose production and release is a key factor in regulating overall lipid metabolism.

Lipids

Obesity and diabetes are associated with dysregulation of hepatic lipid synthesis. Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) are hepatic lipogenic enzymes whose gene transcription is regulated by cellular nutrient levels (Towle et al. 1997). Fatty acids are synthesized when cellular energy is in demand and are either utilized immediately or combined with glycerol to form TG to be stored as neutral lipids. Neutral lipids, including triacylglycerols (TG), steryl esters (SE), and wax esters (WE), are lipids which are unable to penetrate phospholipids bilayers (Athenstaedt and Daum 2006).

Sterols, free fatty acids, and diacylglycerols (DAG) are stored intracellularly after conversion to TGs and SEs. TGs are the major form of lipid storage within tissues, and the fatty acids stored as TG may be oxidized by β-oxidation to produce cellular energy. Therefore, regulation of the release of fatty acids from their neutral lipid storage form may be key in regulating metabolic expenditure.

Neutral lipids are stored in intracellular compartments called lipid droplets (LD). The lipid droplet consists of a phospholipid monolayer, with just a few embedded proteins that control the extraction and hydrolysis of lipids from LDs, and a hydrophobic core containing the neutral lipids (Athenstaedt and Daum 2006). Lipases and hydrolases regulate the release of neutral lipids from the cores of LDs. These neutral lipids can then be broken down to fatty acids that can be utilized by the cell. The free fatty acids and DAG are oxidized primarily for energy.

The exact mechanisms of how LDs form are still under investigation. Van Meer (2001), Ostemeyer et al. (2001), and Murphy et al. (1999) propose that as neutral lipids are being synthesized between the two leaflets of the endoplasmic reticulum (ER), they accumulate due to their

inability to pass through the ER membrane. Once the neutral lipids reach a certain size, they bud off one leaflet of the ER and enter the cytosol as a lipid droplet. Once lipid droplets are formed, they can increase in size by *de novo* synthesis of neutral lipid using enzymes embedded in the phospholipid monolayer and/or within the core of the LD (Athenstaedt and Daum 2006). The core contains the necessary components necessary to make the precursors that form TG. Pathologies associated with obesity and diabetes arise in part because of problems accessing neutral lipids in the LDs to extract the fatty acids. This problem results in the accumulation of excess lipids and larger LDs within the liver and adipose tissue (Greenberg et al. 2006).

Adipose Tissue and Triacylglycerol Hydrolysis

Adipose tissue is not only a major storage depot for excess fat, but more recently it has been found to be an endocrine organ involved in the regulation of whole-body metabolism (Mohamed-Ali et al. 1998). While leptin is secreted from adipose tissue, other hormones such as thyroid hormone may stimulate adipose tissue to further regulate metabolism. Leptin and thyroid hormone regulate the activity of adipose lipolytic enzymes and thus control the hydrolysis of TG within adipocytes. Hormone sensitive

lipase (HSL) is the best-studied adipose lipase and works to induce lipolysis via a cyclic-AMP (cAMP)-meditated phosphorylation cascade that translocates HSL to the surface of LDs where it can then access and hydrolize the newly released TG into glycerol and fatty acids. In addition to HSL, other lipases may be activated by other cell signals that regulate the same process. For instance, deletion of the murine HSL gene only decreased half of the TG lipase activity in adipocytes, suggesting that at least one other enzyme also facilitates this process (Wang et al. 2001; Osuga et al. 2000). Triacylglycerol hydrolase (TGH) is also found in adipose tissue (Dolinsky et al. 2001). W. Gao and R. Lehner showed that reducing murine TGH expression reduced adipocyte free fatty acid release and caused a 40% decrease in plasma free fatty acids (Athenstaedt and Daum 2006). More recently, adipose triglyceride lipase (ATGL) was discovered by Zimmerman et al. (2004), and they demonstrated that ATGL is responsible for 70% of TG lipase activity in adipose tissue, an apparent conflict with previous reports on HSL and TGH activity. This result suggests that the activity of HSL and TGH may have been overestimated and that ATGL might be just as important for lipolysis. More work is needed to examine

the possibility that the activity of any of the lipases is affected by the absence or presence of the other ones.

The hydrolysis of TG occurs in response to a signal released from the SNS that fatty acids are needed for metabolism. These fatty acids are secreted into the bloodstream for circulation to their target tissues, primarily skeletal muscle and liver. If they are not needed in these tissues or cannot be taken up due to insulin insensitivity, they may remain in circulation for longer periods of time. The misregulation of the release and hydrolysis of TG is a critical factor in the development of insulin resistance, hyperinsulinemia, hyperglycemia, and dyslipidemia because glucose uptake into muscle and liver is also altered when there is a chronic increase in circulating free fatty acids (Boden and Shulman 2002; Boden 2003). Thus, the genes and proteins that control the activity of HSL, TGH, and ATGL are possible links to the observed defects in the obese syndrome that leads to insulin resistance.

Thyroid Hormone

The thyroid gland is an important organ that is involved in various physiological processes. A negative feedback loop that involves the hypothalamus, pituitary,

and thyroid gland controls the synthesis and secretion of thyroid hormones (Shupnik et al. 1989). Thyroid hormones are derived from the amino acid tyrosine. The two main types of thyroid hormones are 3,5,3′,5′-tetraiodo-$_L$-thyronine (T_4) and 3,5,3′-triido-$_L$-thyronine (T_3), which differ by the number of iodide ions they contain. Iodide is taken up by the follicular cells of the thyroid gland via a Na^+-I^- co-transport system driven by the plasma membrane Na^+ gradient and becomes oxidized to an active form. This reactive iodide reacts with specific tyrosine residues within thyroglobulin (Tg, protein produced by thyroid gland) via oxidative coupling to form either 3-monoiodotyrosine (MIT) or 3, 5-diiodotyrosine (DIT). If two DIT moieties are joined together, then T_4 is produced. If one MIT and one DIT moiety are joined, then T_3 is produced. The signal to produce thyroid hormones occurs when the pituitary gland releases thyroid stimulating hormone (TSH), which stimulates intracellular cAMP production in the follicular cells of the thyroid gland, leading to the production of T_4 and T_3. Tg contains MIT, DIT, T_3, and T_4 and is stored as a colloid droplet within the follicular lumen for immediate release when needed. To release T_4 and T_3, Tg undergoes endocytosis from the lumen at the apical surface

of the follicular cell and is digested inside the cell to release the hormones, which then diffuse into the blood vessels for circulation to their target tissues (Taurog 1996).

Total serum T_4 is 40-fold higher than total serum T_3; however, T_4 is not as widely used by target tissues as T_3, but both T_4 and T_3 have been shown to produce physiological responses. Most of the serum T_3 and T_4 are bound to thyroxine binding globulin, albumin, or thyroid binding prealbumin when in an inactive form. Thyroid hormones must be released from the protein carriers to enter target cells to elicit biological responses. T_3 is involved in numerous physiological processes and is in higher abundance in the free form than T_4 (Yen 2001). However, even though free T_3 is more abundant than free T_4, most of the T_3 effects are exerted when T_4 is converted to T_3 locally at the tissues (Yen 2001). At the liver and kidney, T_4 is converted to T_3 by type I deiodinase, and this accounts for most of the T_4 turnover observed in circulation (Braverman et al. 1970; Kohrle 2000). Additionally, T_4 is converted to T_3 intracellularly by type II deiodinase in the brain, pituitary, and brown adipose tissue.

Both thyroid hormones activate transcription of specific genes by binding to a member of the thyroid receptor (TR) family located in the nucleus of cells of various organs. When T_3 is bound to TR, gene transcription is activated through thyroid response elements (TRE), which are sometimes pre-bound to TRs, located on the promoter regions of target genes. When T_3 is not bound to TRs, there is repression of gene transcription (Zhang and Lazar 2000).

Thyroid Hormone and Adipose Tissue

Adipocyte differentiation has been shown to be induced by T_3. Grimaldi et al. (1982) demonstrated that preadipocytes from young rats, as well as cells from preadipocyte cell lines, were induced to become white adipose tissue when treated with T_3, as shown through stimulated intracellular lipid accumulation, transcription of key lipogenic enzymes, and adipocyte cell proliferation. The lipogenic enzymes that are induced include ATP-citrate lyase, malic enzyme, and fatty acid synthase, all of which can lead to the synthesis of fatty acids (Blennemann et al. 1995; Gharbi-Chihi et al. 1991).

Thyroid hormones indirectly regulate lipolysis in adipocytes by enhancing the binding of catecholamines to β-adrenergic receptors on adipocytes; thyroid hormones

increase β-adrenergic receptor density and decrease phosphodiesterase activity (Engfeldt et al. 1982; Wahrenberg et al. 1986). β-adrenergic signaling activates adenylate cyclase and mediates a signal transduction cascade via the second messenger cAMP, leading to the activation of lipases that catalyze the breakdown of triacylglycerol (via HSL) into fatty acids and glycerol. Therefore, lipid metabolism can be altered depending upon thyroid status.

The quantity of circulating thyroid hormones may be altered due to defects in the feedback loop that controls thyroid hormone secretion. Hyperthyroidism and hypothyroidism occur when there is over secretion and under secretion of thyroid hormones from the thyroid gland, respectively. During a state of hyperthyroidism, hyperplasia (increase in cell number) and an increase in lipolysis occurs in adipocytes (Levacher et al. 1984). Hypothyroidism, on the other hand, results in hypertrophy (increase in cell size) and decreased lipolysis in adipocytes, resulting in less available free fatty acids for metabolic use. Furthermore, hypothyroidism is associated with low metabolic rate, low body temperature, slow heart rate, and weight gain (Hadley 2000). Therefore,

during hypothyroidism, adipocytes are expected to increase in cell size when compared to a euthyroid state (normal thyroid function) because the cells are saving energy due to the effects of low T_3.

Thyroid hormones regulate genes involved in energy utilization, lipolysis, and lipogenesis. However, these processes do not all occur in the same time frame. In a study of chronic T_3 injections in adult rats (Oppenheimer et al. 1991), total oxygen consumption, which is a measurement of metabolic rate, reached its peak in only 4 days of treatment, and by the sixth day, the amount of total body fat storage dropped by half. However, caloric intake did not reach its peak until day 8, after continually increasing during the previous treatment days. The animals probably still lost body fat despite increased caloric intake because metabolic rates were increased as well, and the calories were needed to maintain energy levels that were being depleted due to increased lipolysis. Furthermore, transcription of genes needed for thermogenesis, lipolysis, and lipogenesis were upregulated by thyroid hormone before they were even needed. This suggests that thyroid hormone-stimulated lipolysis is not directly linked to thyroid hormone-stimulated energy

utilization because genes needed for lipolysis are upregulated as a default mechanism, and this occurs before energy is required.

Thyroid Hormone and Liver

Thyroid hormones can also have multiple effects on the liver. For instance, malic enzyme is a lipogenic enzyme found in the liver, as well as other tissues. In a study by Strait et al. (1989), the transcription of hepatic malic enzyme showed biphasic regulation, as expression increased at 4 hours and again at 24 hours in response to T_3 treatment. This suggests that while T_3 has initial direct effects, secondary effects caused by genes initially upregulated by T_3 also elicit the same type of response as T_3 on its target tissues. The specific effects of T_3 on its target tissues can be dependent upon carbohydrate intake, insulin, and cAMP (Oppenheimer et al. 1987, 1991). Because of these variables, there needs to be consistency in experimental design, using either fasting or non-fasting animals, because the presence or absence of food can alter the activity of T_3. For instance, in fasted animals, T_3 has only minimal effects on the induction of malic enzyme, whereas animals fed a high carbohydrate diet show the

highest levels of T_3-stimulated malic enzyme lipogenesis (Oppenheimer et al. 1987, 1991).

Thyroid hormone regulates the expression of about 8% of hepatic genes *in vivo* (Oppenheimer et al. 1987). Feng et al. (2000) used a cDNA microarray to identify the hepatic genes that are regulated by thyroid hormone. In hypothyroid mice treated with T_3, microarray analysis revealed that 14 genes were up-regulated and 41 were down-regulated. These included genes involved in various cellular processes, including increasing glycogenolysis, gluconeogenesis, lipogenesis, cAMP activity, β2-Adrenergic receptor activity, proliferation, and apoptosis. It also included genes that decreased insulin action, immunogenecity, and protein glycosylation.

The *ob/ob* Mouse Model

With failed attempts to treat obese human patients with leptin injections (Houseknecht et al. 1998a), there has been an increasing need to understand the mechanisms involved in lipid trafficking in order to determine appropriate routes for therapy. Since leptin reduces appetite and lowers fat mass in mice, it is inconclusive as to why treatment with leptin does not work as a therapeutic agent in humans. Abnormalities in other hormone

concentrations that occur in obesity may hinder the action of leptin. Leptin may possibly interact with these other hormones to affect lipid trafficking, making the determination of its actions alone more difficult. There are many interacting physiological processes that occur at any given time in the human body. Certain available animal models may be useful in isolating certain metabolic pathways and determining the mode of action of a single hormone.

There are two well-known mouse models used to study obesity and diabetes. They are the obese (*ob/ob*, described below) and diabetic (*db/db*) mice. The *ob/ob* mouse is leptin deficient, but this deficiency can be rescued by injection of leptin because leptin receptors, particularly in the brain, are still responsive to leptin (Zhang et al. 1994). The leptin receptors of *db/db* mice are nonfunctional, thus the injection of leptin has no effect, i.e. the mice are leptin resistant, which seems to be the case for most humans (Ghilardi et al. 1996). Therefore, in order for the effects of exogenous leptin on obesity to be observed, the *ob/ob* mouse model needs to be used.

The mouse *ob* gene codes for the protein leptin. This protein has an amino acid sequence 84% identical to that

found in humans (Zhang et al. 1994). The C57BL/6J *ob/ob* mouse strain was first isolated by Ingalls et al. (1950) and was later characterized by Zhang et al. (1994). They found *ob/ob* mice express a twenty-fold increase in abnormal *ob* mRNA that contain a single base substitution resulting in a nonsense mutation. This substitution results in a truncated leptin protein, leading to leptin protein deficiency. The lack of leptin leads to morbid obesity, hyperphagia, hypothermia, extreme insulin resistance, infertility, and many other endocrine abnormalities (Ingalls et al. 1950). The lack of an appetite inhibition signal associated with chronic leptin deficiency can cause the brain to perceive a starvation status even though there are adequate circulating and stored carbohydrates and lipids. Thus, the brain continuously stimulates appetite and food intake, which leads to obesity (Elmquist et al. 1998b). The *ob/ob* mouse model is useful in investigating how defects in lipid metabolism (due to reduced concentrations of circulating leptin levels) lead to serious conditions such as insulin resistance.

The roles of thyroid hormone in regulating physiological pathways involved in metabolism, including lipolysis, and leptin's role in maintaining the balance

between circulating nutrient levels and the quantity stored in various tissues, suggest these two hormones may interact to affect lipid storage. However, it is not known if the actions of leptin are dependent on the presence of thyroid hormones. T_3 concentrations can be lowered by the drug iopanoic acid (IOP), which blocks the activity of type I deiodinase, allowing the possibility to study leptin's effects in the presence of decreased T_3 levels.

Previous Work in Dr. Richard Fehn's Lab

The interactions of thyroid hormone and leptin have been studied in Dr. Fehn's lab using both the *ob/ob* and *db/db* mouse models to evaluate histological and molecular data from various tissues. In a study by Brian Underhill (2000), C57BL/6J *ob/ob* mice were treated for nine days with saline vehicle, leptin, IOP, or leptin/IOP. He monitored daily food consumption, body weight, body heat production (measured by radiant body temperature), oxygen consumption, respiratory quotient (RQ), and circulating T_3 and T_4 levels. Results revealed that leptin and leptin/IOP treatments caused a reduction in food consumption, while IOP treatment showed no change. The decrease in body weight was lowest in leptin/IOP treated mice, with leptin treatment still causing weight loss when compared to vehicle treated mice,

and IOP treated mice showing no change. Body heat production was increased with leptin/IOP treatment as compared to vehicle treatment, but mice treated with leptin and IOP alone showed body heat production equal to that of vehicle treated mice. Oxygen consumption rates (V_{O2}) for leptin and leptin/IOP treated mice were the same as the vehicle treatment. However, IOP treated mice had a lower V_{O2} compared to the vehicle treated mice. This suggests that leptin prevented the reduction in oxygen consumption observed in the IOP treated mice. Furthermore, mice in all treatments expressed RQ values representative of lipid metabolism. Finally, T_3 levels were significantly elevated in *ob/ob* vehicle treated mice compared to *ob/+* vehicle treated mice, but the remaining three treatment groups in *ob/ob* mice showed no difference among one another. Taken together, these results show that reduced body weight in leptin and leptin/IOP treated mice may be due ultimately to decreased food consumption observed in these groups. IOP treated mice may weigh equal to that of vehicle treated mice because of the similar food consumption between the two groups.

The observation that all of these mice may have been primarily utilizing lipid to fuel metabolism may have been

erroneous. Underhill's results were in contrast with a later study by Reno and Fehn (2007) that found that vehicle and IOP treated *ob/ob* mice had an RQ representative of carbohydrate metabolism, while leptin treated mice had an RQ representative of lipid metabolism. It was also suggested by Reno and Fehn (2007) that the lipid metabolism by any animal with leptin treatment may have been necessary to support their increased production of body heat, which is indicative of higher energy expenditure through thermogenesis. On the other hand, increased thermogenesis could also have been a result of increased lipid metabolism.

During periods of increased energy expenditure, lipid is the preferred metabolic substrate since lipid is stored and readily available for use. All of the animals in both studies started out obese, and, therefore, had abundant lipid available for use, possibly supporting the notion that all animals used lipid metabolism, but Underhill's results do not follow the pattern that was seen in the later study (Reno and Fehn 2007). For instance, Reno and Fehn (2007) saw an increase in lipid metabolism and suggested this could support an increase in body heat production observed during leptin treatment. Also, the

radiant body temperatures in Underhill's study showed that leptin/IOP treatment increased body heat production, while the other three treatments showed no change. Therefore, his results demonstrated that if body heat production is one factor that can alter which substrate is used in metabolism, then the vehicle, leptin, and IOP treated mice should have been using carbohydrates as a metabolic substrate if there was no increase in energy as heat. However, with the exception of IOP treated mice, the oxygen consumption rates were relatively constant among the treatment groups. Taken together, the results of both studies reveal the importance of not one, but many factors that underlie whether carbohydrate or lipid metabolism is preferred. Moreover, mice may switch between carbohydrate and lipid use in a daily cycle depending on whether they are active or sleeping (unpublished data from Dr. Fehn's lab).

Another interesting contradiction was found between previous studies reported in the literature and research in Dr. Fehn's lab in regards to the effects of leptin treatment. Pelleymounter et al. (1995) state that *ob/ob* mice of the C57BL/6J strain given leptin increase oxygen consumption, while Dr. Fehn's lab (Reno and Fehn 2007;

Underhill 2000) found no increase in leptin treated mice compared to vehicle treated mice. Pelleymounter et al.'s study was done on 5-week old mice over a 28 day injection period, while the Fehn studies were done on 10-12-week old mice over a 9 day injection period. It may be inferred that a longer treatment period with leptin may have additional affects, but Fehn's lab showed that after nine days of treatment, all parameters measured in the mice reach a peak and do not go any further. Age effects may also account for the differences observed between the studies. For instance, the onset of obesity in mice occurs around 5 weeks of age and 10-12-week old mice are considered to be reproductively mature. My current study will help clarify whether or not leptin injection stimulates significant increases in metabolism and what effects an increased metabolism may have on other measurable parameters such as metabolic substrate utilization.

Due to the importance of these factors in evaluating the end point analyses of hepatic lipids and adipocytes, all of the afore mentioned metabolic parameters need to be assessed again in my project. I expect the results in my experiments to be a mix of those which found in literature (Pelleymounter et al. 1995) and those found in the Fehn lab

studies (Reno and Fehn 2007; Underhill 2000) (table 1). My study on hepatic lipid content and adipocyte area (described below) will provide additional data on the relationship between leptin and thyroid hormone in lipid metabolism.

Preliminary Work and Hypotheses

Previous work has been done on C57BL/6J *ob/ob* mice where these mice were treated for nine days with vehicle, leptin, T_3, IOP, 6-hydroxydopanine (6OHDA), leptin/ T_3, Leptin/6OHDA, T_3/6OHDA, and T_3/IOP (Reno and Fehn 2007). Analysis of hepatic lipid content and lipid droplet size by transmission electron microscopy (TEM) and light microscopy revealed that treatment groups lacking leptin contained more hepatic lipid droplets and larger lipid droplets, suggesting lipid storage. In animals treated with leptin/6OHDA and leptin/T_3, hepatic lipid droplet size and content were significantly decreased. (Content was assessed by cross-sectional area of hepatocytes.) This suggested that leptin is very important in facilitating the breakdown and export of stored and newly synthesized lipids because it is able to produce its responses even under adverse conditions. These were adverse conditions because sympathetic neurons are important in energy homeostasis and

Table 1. Expected Results of Parameters to be Tested.

	Vehicle	Leptin	IOP	Leptin/IOP
Oxygen consumption rate	----	↑	↓	----
Body weight	----	↓	----	↓
RQ	CHO	Lipid	CHO	Lipid
Food consumption	----	↓	----	↓

IOP treated mice should have the lowest oxygen consumption rate while leptin treated mice should have the highest. The mice in the remaining two treatment groups should show rates of equal value. Body weight should be reduced in leptin and leptin/IOP treated mice, while IOP treated mice should have body weight equal to that of vehicle treated mice. RQ values should reveal that carbohydrates are being utilized as a metabolic substrate in the vehicle and IOP treated mice, but mice from the other two treatment groups that receive leptin should show RQ values representative of lipid metabolism. Food consumption should be reduced in leptin and leptin/IOP treated mice, while IOP treated mice should eat about the same amount as vehicle treated mice. ---- = no change; ↓ = decreased values when compared to vehicle; CHO= carbohydrate

hyperthyroidism can lead to further metabolic complications. Surprising results were obtained when evaluating omental and subcutaneous adipocyte volumes: leptin treated mice (alone and combined treatments) had the largest adipocyte areas compared to all of the other treatment groups. This was interesting given that leptin

treatment reduces body weight. It was therefore assumed that adipocytes would be smaller than adipocytes from an obese mouse given vehicle treatment. One explanation for the observed results is that the liver was exporting a vast amount of stored lipids and sending them to adipose tissue because storing lipids in adipocytes is a more efficient way to store excess lipid. This idea is supported by the decreased liver lipid content, decreased liver weight, and stable glycogen levels in the liver. The stable glycogen levels suggest that liver weight was lost not due to an increase in metabolism but due to the export of lipids, which then moved to adipocytes. This seems logical because storage of excess lipid in the liver leads to serious problems, such as liver failure and hepatic steatosis (displacement of the cytoplasm and organelles due to excess lipid accumulation), and adipose tissue is meant to store lipid. In this study, total oxygen consumption did not increase with leptin treatment, suggesting less lipid disposal from adipocytes for metabolism.

Another interesting observation was that IOP treatment alone caused massive hepatic steatosis. Analysis of hepatocytes under TEM showed apoptosis likely due to enlarged lipids distorting organelles, including the

nucleus. Interestingly, when IOP treatment was combined with T_3, large hepatic lipid droplets were still prevalent whereas mice treated with T_3 alone had numerous small lipid droplets. Therefore, the low T_3 concentration in IOP treated mice may not be the only underlying factor as to why hepatic lipids are so large since T_3 replacement in a T_3/IOP combined treatment still exhibited large lipid droplets.

One question that arose was if leptin can cause a reduction in hepatic lipid droplet content and size in animals that have SNS neuron ablation (Leptin/6OHDA) and in animals with excess lipid synthesis (leptin/T_3), then leptin should be able to breakdown large lipid droplets and export them even during the massive hepatic steatosis observed in IOP treated animals. Thus, a combined treatment of leptin/IOP could reveal whether or not low T_3 concentration has an effect on hepatic lipid transport even in the presence of leptin, which as mentioned above has been shown to clear the liver of excess lipid under various adverse circumstances. If leptin does not decrease hepatic lipid size and content in the presence of IOP treatment, then T_3 or some other factor regulated by T_3 may be important in helping to facilitate the breakdown of lipids in the liver. If leptin does decrease hepatic lipid size and content in

the presence of IOP treatment, then T_3 is not important in aiding leptin to export lipids, supporting the vital importance of leptin being able to function during stressful conditions. It is also expected that adipocyte areas will be large in leptin treated mice and in IOP treated mice, compared to vehicle treated mice, with IOP treated mice possibly having the largest cells. This could be expected because leptin treatment should reduce liver lipid and store it in adipose cells until utilized in metabolism. If oxygen consumption rates are increased in the leptin treatment, then adipocyte areas should be decreased due to an increased use of adipocytic lipid in metabolism. IOP treated mice should have large adipose cells because of the extreme lipid storage in the liver and adipose tissue and decreased oxygen consumption rates previously observed in this treatment. The leptin/IOP treated mice should express smaller adipocytes than vehicle treated mice, but it is not known if leptin can successfully increase metabolism in a low T_3 environment or how the size of those adipocytes will compare to the mice in the leptin and IOP single treatments.

In this thesis, in order to study the effects of leptin under both normal and reduced T_3 concentration,

C57BL/6J *ob/ob* mice were treated for seven days with either vehicle, leptin, IOP, or a combination of leptin/IOP. These treatments should have resulted in mice with T_3 alone (vehicle), leptin + T_3 (leptin treated), neither leptin nor T_3 (IOP treated), or leptin alone (leptin/IOP treated). These mice were analyzed for changes in total mass-specific oxygen consumption, body weight, substrate used in metabolism, and food intake over the seven day period. Additionally, end point analyses determined omental and subcutaneous adipocyte area and hepatic lipid content and LD size.

CHAPTER TWO

THE EFFECTS OF LEPTIN AND THYROID HORMONE ON METABOLISM AND LIPID TRAFFICKING IN THE LIVER AND ADIPOSE TISSUE OF *OB/OB* MICE

Introduction

With obesity becoming a world-wide epidemic, it is imperative to understand the pathology associated with obesity. Obesity is result of the altered control of hormones regulating metabolic glucose and lipid trafficking pathways (such as leptin and thyroid hormone)(Houseknecht 1998). The actions of these hormones are targeted as possible routes of therapy to control obesity and its complications. Leptin is a hormone secreted by adipose tissue in response to fluctuation in cellular energy reserves (Tartaglia et al. 1995). Its main role is to act as an appetite inhibitor when enough "energy" has been taken in. Leptin deficiency would reduce feeding inhibition and, therefore, promote obesity. The thyroid hormone 3,5,3'-triido-$_L$-thyronine (T_3) regulates lipogenesis, lipolysis, fat storage, and basal oxygen consumption rate(Oppenheimer 1991). Since T_3 induces an increase in

metabolism, which primarily utilizes fatty acids stored in adipose tissue, the hormone also induces the production of lipids, allowing a reserve to be maintained for the increased oxygen consumption rate that results from the increased metabolism (Oppenheimer 1991). Low levels of thyroid hormone can lead to a decrease in lipolysis, which would lead to an increase in body fat stores in adipose tissue, and low T_3 is associated with low metabolic rate, low body temperature, slow heart rate, and weight gain (Hadley 2000). Leptin and thyroid hormone are thought to interact to control energy homeostasis, but the exact interaction(s) is unknown.

Pathologies associated with obesity and diabetes are often linked to leptin deficiency or leptin resistance. The *ob/ob* mice of strain C57BL/6J are leptin deficient and thus provide a good model to investigate abnormalities related to leptin deficiency. Exogenous leptin replacement in *ob/ob* mice has been shown to increase total body energy expenditure, decrease food intake, and normalize body weight (Halaas et al. 1995; Maffei et al. 1995). However, the exact mechanisms leading to these responses are poorly understood.

Thyroid hormone regulates physiological pathways involved in metabolism (including lipid metabolism), whereas leptin acts to maintain the balance between circulating lipid and glucose levels and the quantity stored in various tissues. This suggests that these two hormones may interact to affect lipid storage and disruption of this interaction may lead to the development of obesity. In this thesis, the possible cross-effects of leptin and thyroid hormone in obesity were studied in leptin deficient *ob/ob* mice. Individuals were administered iopanoic acid (IOP) to reduce thyroid hormone concentrations, and leptin, to rescue leptin deficiency. Reducing T_3 will allow us to assess leptin's ability to increase body energy expenditure, decrease food intake, and normalize body weight in the presence of low T_3 concentrations. The following changes were assessed in mice given leptin, IOP, or a combination of both: oxygen consumption, respiratory quotient values, food consumption, percent body weight change, omental and subcutaneous adipocyte sizes, and hepatic lipid droplet content and size.

Methods

Twenty 9 week old female mice, strain C57BL/6J *ob/ob* (Jackson Laboratory), were used to analyze variation in metabolic expenditures in response to four different treatments. Five animals per treatment group were used for data analysis (previously shown to be an adequate sample size for statistical significance).

The mice were housed individually in a 12:12 light:dark cycle and allowed access to food *ad libitum*. On the morning of day zero, all mice were given a known quantity of normal rodent chow (4% fat). Variation in food intake during the treatment period was calculated by subtracting the amount of food remaining from the previous day's amount. Each mouse was weighed and placed into an individual chamber of a flow-through metabolic system (AMETEK, AEI Technologies; S-3A/II oxygen and CD-3A carbon dioxide analyzers). Air was pulled out of each chamber at 400 ml/min and analyzed for the fraction of oxygen and carbon dioxide content. Excurrent air was passed through desiccant to remove any moisture. Each mouse was allowed to settle in the chamber for five minutes prior to data collection. Readings for each animal were taken in the morning (between 06:00- 09:00 hours). Each animal was

sampled three times over a 24 minute period, and the average value was used in subsequent analyses. During the reading period, the mice were observed for relative activity levels that may have affected metabolic readings and any behavioral responses to the treatments were recorded. Mass specific metabolic rate (total oxygen consumption) was calculated as:

$$V_{O2} = \frac{(F_{O2}\ \text{in} - F_{O2}\ \text{out})\ V}{Mb}$$

- V_{O2} = the measurement of oxygen uptake, expressed as a volume of consumed O_2 per minute per gram body mass,
- F_{O2} in = fraction of O_2 in the air entering the chamber,
- F_{O2} out = fraction of O_2 in the air exiting the chamber,
- V = the flow rate of air moving through the chamber (ml/min)
- Mb = body weight (g)

Respiratory quotient (RQ) is a measure of metabolic substrate use and can range from 0.71 (lipid utilization) to 1.0 (carbohydrate utilization); an RQ of around 0.80 suggests protein utilization. The RQ value is affected by the differences in the amount of energy required to consume

a lipid, protein, or carbohydrate, as well as the chemical balance of carbon, hydrogen, and oxygen atoms in the consumption equation. The RQ was calculated as:

$$RQ = (V_{CO2}) / (V_{O2})$$

- V_{CO2} = CO_2 production rate
- V_{O2} = O_2 consumption rate.

Eight channels on the gas analyzer allowed eight mice to be sampled at the same time on the same day which allowed for increased efficiency in gathering results as well as consistency. Due to the relatively constant conditions in the animal house, readings done during separate time frames would not affect the results because they were both done in the morning.

There were five *ob/ob* leptin deficient mice per group in each of the following treatments: vehicle (control for handling and injecting mice), leptin (to rescue leptin deficiency), IOP (to effectively lower concentrations of T_3), and leptin/IOP (to observe the affects of leptin in the presence of low T_3). (See appendix B for recipes.) Each animal was injected intraperitoneally (I.P.) with its respective drug treatment after the completion of all metabolic readings.

The metabolic analyses and injections were carried out over eight consecutive days during the same morning hours to control for diurnal variation. Each day, before any analysis was done, each animal was checked to ensure that no adverse affects were seen due to the treatments. If any mouse had symptoms, including extreme exhaustion and listlessness, then it was removed from the colony and dealt with according to IACUC protocols. This was done in addition to observing activity levels during the reading periods.

On the eighth day, all of the afore-mentioned procedures were carried out with the exception of the injections. After morning analyses were complete, all of the mice were euthanized with CO_2 for tissue collection. Blood (ca. 1.0 ml) was collected via cardiac puncture after ventilatory arrest, but just prior to cardiac arrest, and stored on ice until centrifuged after the dissections. The plasma was stored at -80°C. Three pieces (approximately 1 mm^3 each) of the lower left lobe of the liver from each mouse were extracted and fixed in 2.5% glutaraldehyde with Tyrode's Cacodylic acid for TEM embedding (see appendix C). The remainder of the liver was frozen and stored in liquid nitrogen. Omental and subcutaneous adipose tissues were

frozen and stored in liquid nitrogen until analysis of cell cross-sectioned area. Small pieces (size irrelevant) of the omental and subcutaneous adipose tissues were taken, placed on a microscope slide, stained with methylene blue, and examined and photographed using light microscopy at 200x magnification. Multiple images were captured per slide using NIH Image and later analyzed for adipose cell areas using ImageJ. Ten cells per tissue were analyzed, and the average cell cross-sectioned area was taken as representative.

Size and distribution of liver lipid droplets were analyzed using the TEM embedded tissues. The osmium tetroxide fixative stains the lipids black and allows for analysis of lipid content. The tissues were sectioned to a thickness of 900 nm using an ultramicrotome. The sections were placed on a microscope slide and examined using light microscopy at 400x and 1000x magnification. NIH Image was used to capture three images per slide per magnification, and ImageJ was used to determine lipid droplet sizes and content in hepatocytes.

All data were analyzed using one way analysis of variance (ANOVA) in Sigma Stat (Systat Software, Inc. 2004). Where relevant, nested ANOVA was performed, i.e.

lipid droplet size was modeled by group and mouse. This allowed the data from three separate cross-sections per mouse to be analyzed for group effects more efficiently. Pairwise comparisons were done with Tukey's test. Dunn's test was used to compare multiple points against a control group. Linear regression analyses were performed between all variables observed in the study, between groups and within groups between days. Where relevant, linear regressions were run with parameters assessed and food consumption to control for the alterations that might occur based on eating habits. Post-hoc corrections (Bonferroni) were done on all data not subjected to Tukey's test. Data with corrected p values less than 0.05 were considered significant.

Results/Discussion

Daily Observations

The morning of the first day of injections (day 1), all of the twenty mice had high activity levels and were eating normally. By day two, all of the mice in the IOP and leptin/IOP treated groups were lethargic and had stopped eating. Two animals from each of those groups died on days four and five. Since the cause of death from these animals

is not completely certain, all of the data for those individuals were omitted from the analyses. The final sample size per group was as follows: vehicle (veh): 5, leptin (lep): 5, iopanoic acid (IOP): 3, and leptin/iopanoic acid (lep/IOP): 3.

Body Mass

To normalize for differences in body weight among all the mice on day zero (figure 3), body mass change was analyzed by starting at 100 percent on day zero and either adding or subtracting percent change for each treatment group as they either gained or lost weight, respectively. The body mass of vehicle and leptin treated mice did not change whereas IOP and leptin/IOP treated mice lost weight ($p < 0.016$ and $p < 0.008$, respectively; figure 4). The mice in the vehicle group ate steadily every day and showed a correlation of increased body mass with time (see below). The lack of weight gain in mice in the leptin group suggests that leptin inhibited appetite. *Ob/ob* mice do not produce leptin and therefore vehicle treated mice can continue to gain weight. However, the body mass for this group was not significantly greater from day 0.

The relative change in weight between the mice in the leptin treatment and leptin/IOP treatment were

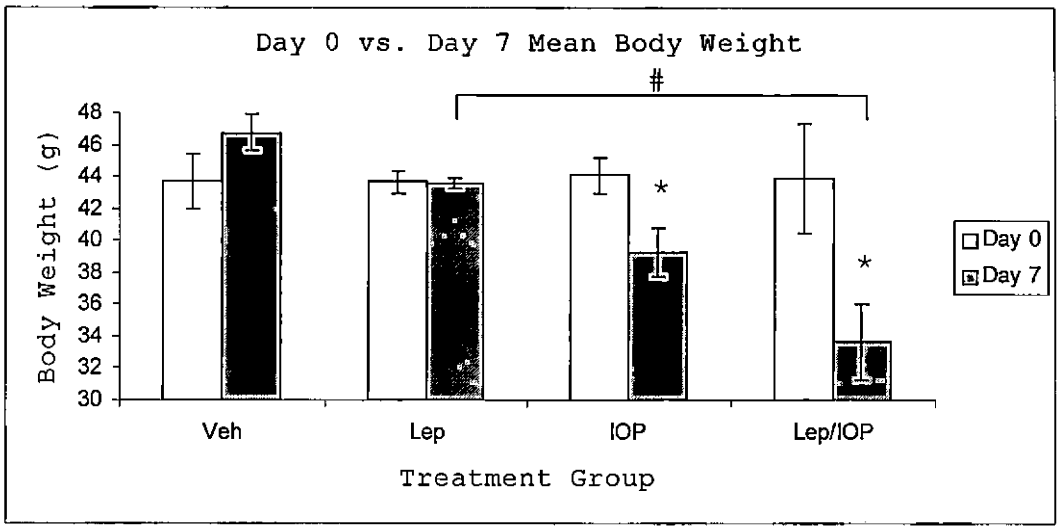
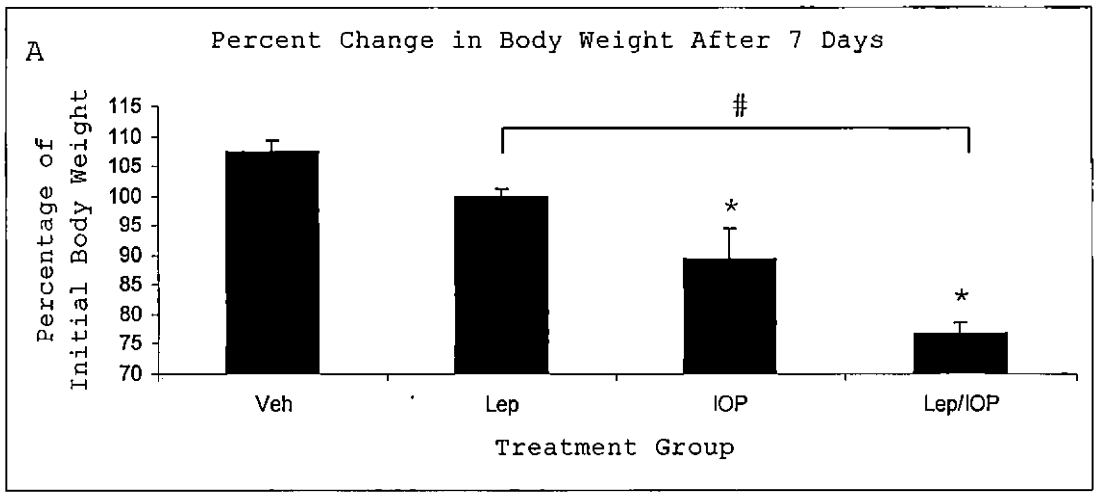


Figure 3. Day 0 vs. Day 7 Mean Body Weight. Statistical analyses show that IOP and leptin/IOP treated mice weighed less on day 7 than vehicle treated mice. Leptin/IOP treated mice also weighed less than leptin treated mice.
*Significant differences in body mass of the groups compared to the vehicle treated group.
#Also significantly different was leptin vs. leptin/IOP; $p < 0.05$
Values are mean ±SEM. For vehicle and leptin, n=5. For IOP and leptin/IOP, n=3.

significantly different from each other after seven days ($p < 0.008$), while no other combinations revealed significant differences among one another. The vehicle and leptin/IOP treated mice showed a significant relationship between change in body mass and day of trial ($r^2= 0.959$, $p< 0.04$ and $r^2= 0.997$, $p< 0.024$, respectively; figure 5). The leptin treated mice did not fluctuate in weight during the study ($r^2= 0.0072$, $p < 0.928$). The IOP treated mice lost weight

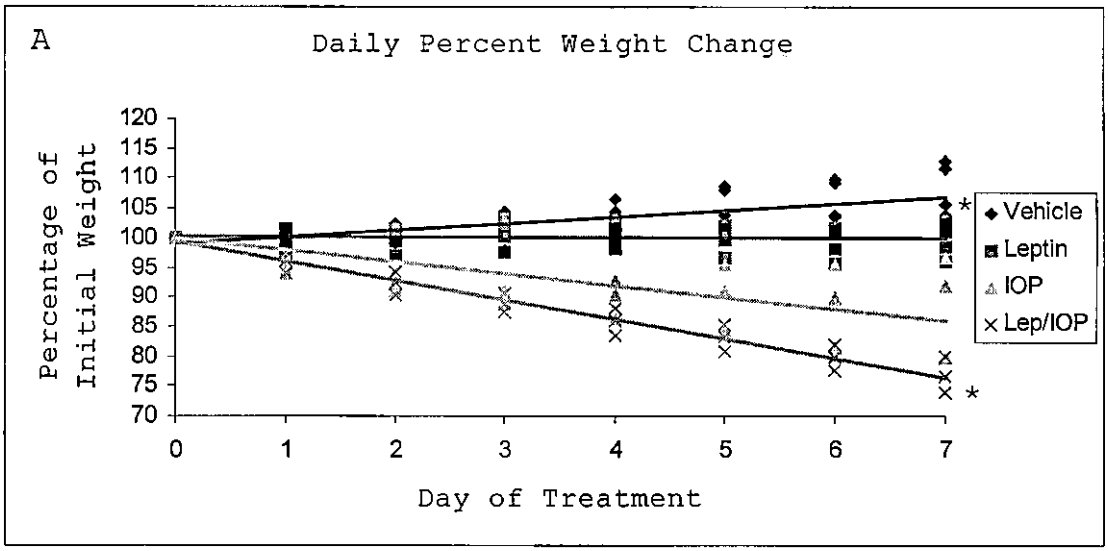


B p values from ANOVA results and Tukey's test

	Vehicle	Leptin	IOP	Leptin/IOP
Vehicle	———			
Leptin	0.110	———		
IOP	0.016	0.392	———	
Leptin/IOP	0.008	0.008	0.228	———

Figure 4. Percent Change in Body Weight After Seven Days of Treatment. Data are normalized to body mass on day 0.
A. Vehicle and leptin treated mice showed no difference in weight change during the treatment period, while the IOP and leptin/IOP treated mice lost weight.
B. p values from ANOVA results and Tukey's Test.
*Significant differences of percent weight change in the groups compared to the vehicle treated group after the treatment period.
#Also significantly different was leptin vs. leptin/IOP; $p < 0.05$
Values are mean ±SEM. For vehicle and leptin, n=5. For IOP and leptin/IOP, n=3.

during the first three days, likely due lack of food consumption associated with treatment, but by day four, two of the three mice still alive recovered and were gaining



B Linear Regression Results

	Vehicle	Leptin	IOP	Leptin/IOP
r^2	0.515	0.00720	0.313	0.950
F	40.4	0.00826	10.0	417
p	0.04*	0.928	0.096	0.024 *

Figure 5. Percent Weight Change Per Day of Treatment.
A. Vehicle treated mice showed a tendency of increasing body weight over the treatment period. Leptin/IOP treated mice showed a tendency of decreasing body weight. Leptin treatment did not affect weight during the treatment period and IOP treated mice showed no tendency of either decreasing or increasing weight. The equations of the lines for the vehicle, leptin, IOP, and leptin/IOP groups were as follows: y= 1.11x + 99.08, y = -0.01x + 100.1, y = -1.27x + 96.18, and y = -3.25x + 99.20, respectively. For vehicle and leptin, n=5. For IOP and leptin/IOP, n=3.
B. Table of r^2, F, and p values from linear regression analyses of daily percent weight change versus day of treatment.

weight. Given that one mouse in this group did not show the same trend and given the small sample size, the body weight

for this group is not strongly correlated with day of treatment (r^2= 0.683, $p < 0.096$).

Food Consumption

Daily food consumption was analyzed to detect the effects of the treatments on the regulation of appetite. Analysis of food consumption began after the first treatment day; therefore, there are no data for pre-treatment food consumption; however, food consumption was determined daily for the time during the treatment.

The body mass normalized average daily food consumption was significantly lower in the IOP and leptin/IOP treated mice relative to control mice ($p < 0.05$; figure 6). Furthermore, the leptin/IOP treated mice consumed less food than the leptin and IOP treated mice. Since the IOP and leptin/IOP treated mice became ill, the decreased food consumption seen in these groups may not be from the treatments directly but due to illness. Some of the animals in the IOP group recovered after day three and started eating again, but one mouse in this group did not eat during the entire treatment period. Therefore, the small sample size (n=3) may be a factor as to why decreased food consumption was observed counter to expectations.

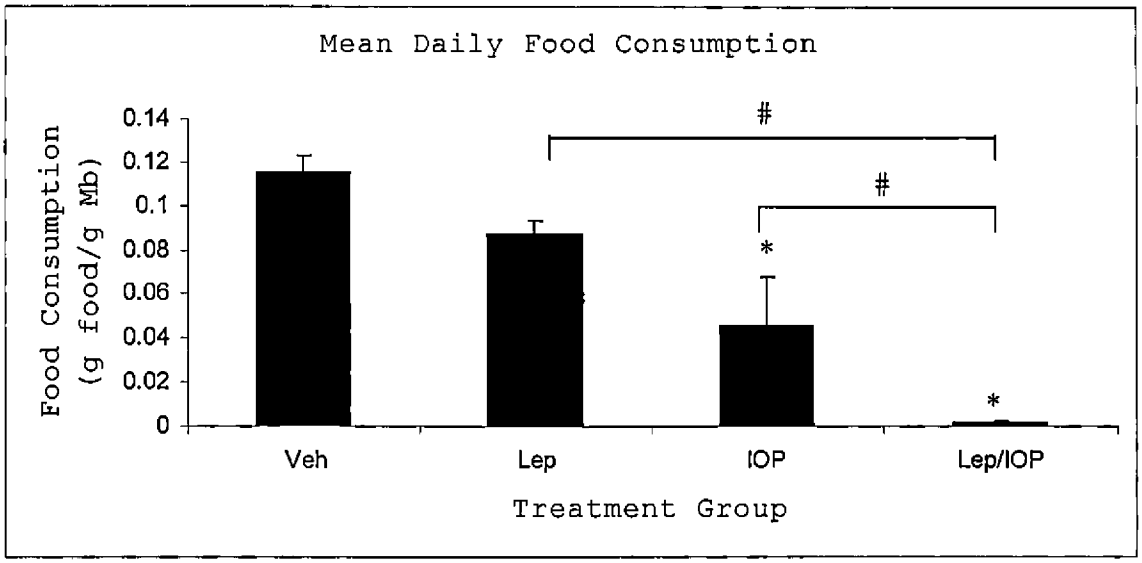


Figure 6. Mass-Specific Mean Daily Food Consumption. Mean daily food consumption per body weight showed that the IOP and leptin/IOP treated mice ate less on average per day than the vehicle and leptin treated mice. Although, a slight decrease in food consumption is seen in the leptin treatment, this was not significant.
*Significant differences with groups against the vehicle treated group.
#Also significantly different were leptin vs. leptin/IOP, IOP vs. leptin/IOP; $p < 0.05$.
Values are mean ±SEM. For vehicle and leptin, n=5. For IOP and leptin/IOP, n=3. MB = body mass weight

The mean food consumption after the seven day treatment period varied between all groups. Leptin treated mice ate less total food than vehicle treated mice, but this was not significantly different. However, total food consumed by both the IOP and leptin/IOP treated mice was significantly less than food consumed by vehicle treated mice ($p < 0.05$, data not shown). On day one of treatment, the IOP and

leptin/IOP treated mice ate less food than vehicle treated mice ($p < 0.05$, figure 7). Only the vehicle treated mice significantly changed their rate of food consumption over the study period ($p < 0.05$, figure 7). However, the remaining IOP treated mice did start eating more after the fourth day of treatment and, therefore, have a higher mean food consumption on day seven. While the difference is not significant between day 1 and day 7 food consumption for IOP treated mice, it can be supported by the relationship between food consumed and day of trial (see below).

Vehicle and IOP treated mice increased mean daily food consumption over the seven day treatment period ($p < 0.005$ and $p < 0.015$, respectively; figure 8). This suggests that the IOP treated mice may have been moving towards the same or greater rate of food consumption as the vehicle treated mice. Therefore, the initial low amount of food consumption seen in the IOP group may have been because the animals in that group were sick and not because of the effects low T_3. The leptin and leptin/IOP treated mice showed no trends towards changing food consumption as a result of their treatment.

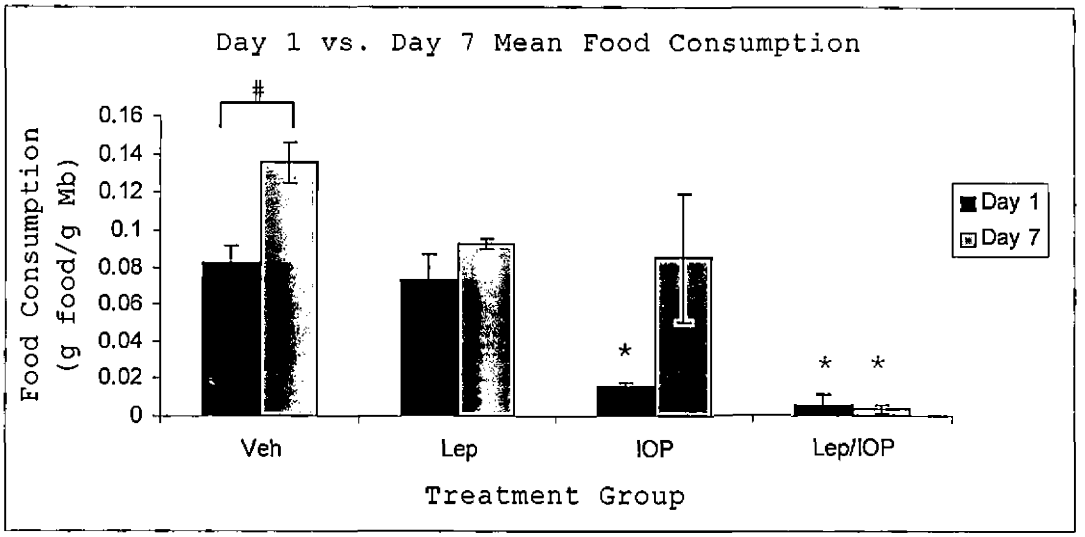
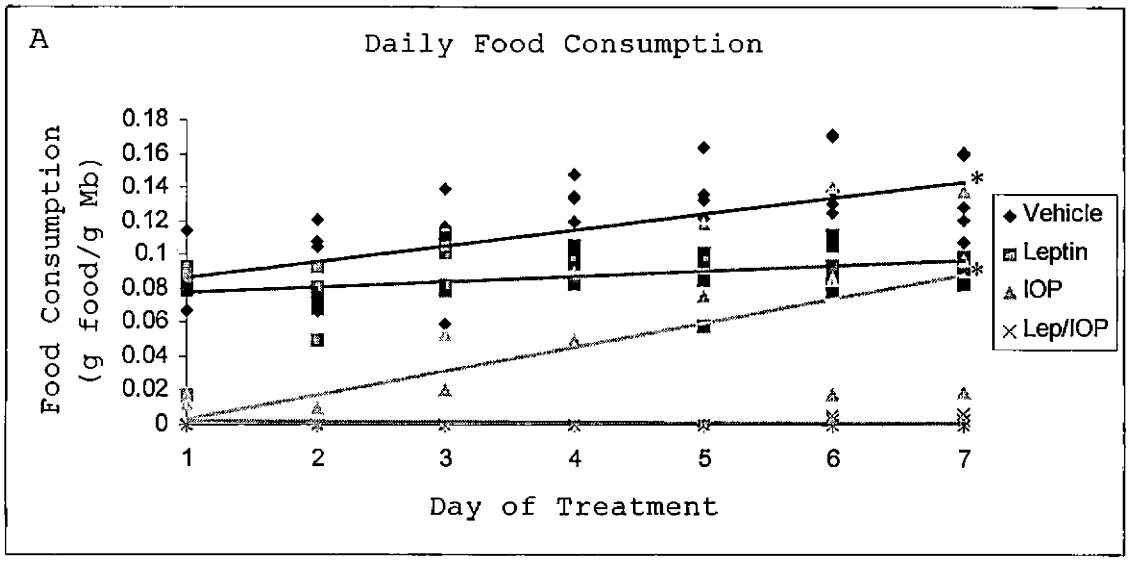


Figure 7. Mean Daily Food Consumption on Days 1 and 7 of the Treatment Period. Compared to vehicle treated mice, the IOP treated mice ate less food on day 1, and leptin/IOP treated mice ate less on day 1 and day 7. On day 7, the leptin treated mice consumed less food compared to the vehicle group, but this was not significant.
#Significant difference between food consumption on day 1 and day 7.
*Significant differences with groups relative to the vehicle treated group for the same day. Also significantly different on day 1 were leptin vs. leptin/IOP, leptin vs. IOP and on day 7 were leptin vs. leptin/IOP, leptin vs. IOP; $p < 0.05$.
Values are mean ±SEM. For vehicle and leptin, n=5. For IOP and leptin/IOP, n=3.

Oxygen Consumption and RQ

Metabolic rates were determined daily using a flow-through metabolic system. There were no differences in mass-specific oxygen consumption between any of the treatment groups on day 7 (figure 9). All groups maintained an approximately constant average rate of oxygen



B Linear Regression Results

	Vehicle	Leptin	IOP	Leptin/IOP
r^2	0.286	0.135	0.372	0.0013
F	13.22	5.135	11.27	0.0247
p	0.005 *	0.150	0.015 *	0.877

Figure 8. Mass-Specific Daily Food Consumption.
A. Trend of average food consumption throughout the seven day treatment period. The vehicle and IOP treated mice showed a tendency of increasing mean daily food consumption, $p < 0.005$ and $p = 0.015$, respectively, while the leptin and leptin/IOP treated groups showed no change over the 7 day treatment period. The equations of the lines for the vehicle, leptin, IOP, and leptin/IOP groups were as follows: $y = 0.0096x + 0.0766$, $y = 0.0033x + 0.0743$, $y = 0.0142x - 0.0106$, and $y = -7E-05x + 0.0019$, respectively.
Mb= body weight
Values are mean. For vehicle and leptin, n=5. For IOP and leptin/IOP, n=3.
B. r^2, F and p values of the treatment groups for linear regression analysis of trends during the seven day treatment period.

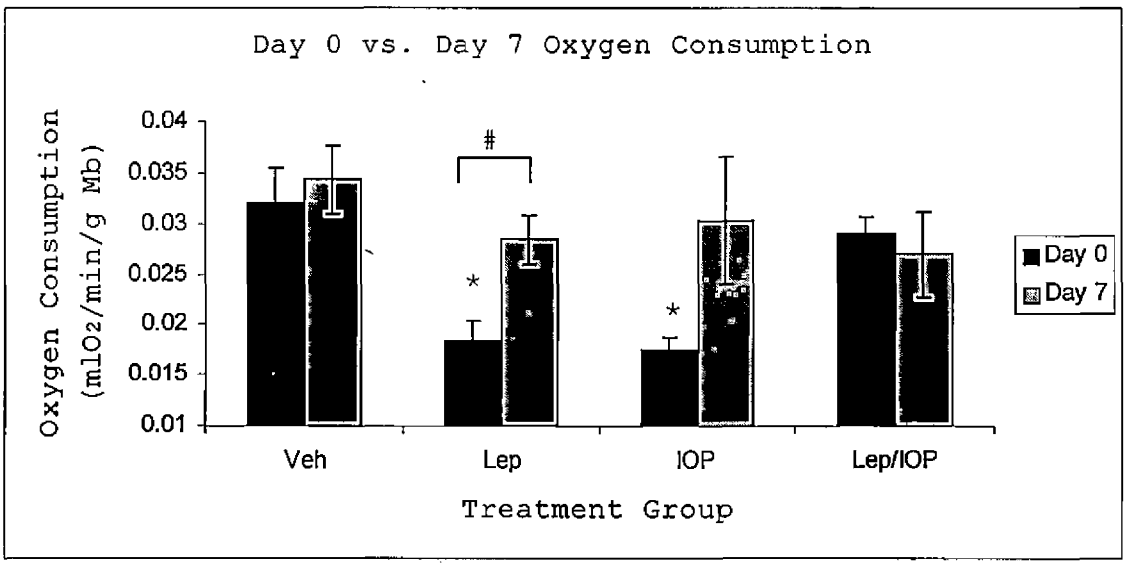


Figure 9. Oxygen Consumption on Days 0 and 7 of the Treatment Period. On day 0, leptin and IOP treated mice consumed less oxygen than vehicle treated mice ($p < 0.007$ and $p < 0.012$, respectively). There were no differences compared to vehicle treated mice for any groups on day 7. Leptin treated mice consumed more oxygen on day 7 than on day 0.
*Significant differences with groups relative to the vehicle treated group for the same day.
#Significance on day 0 vs. day 7 for lep, $p < 0.05$.
Values are mean ±SEM. For vehicle and leptin, n=5. For IOP and leptin/IOP, n=3.

consumption ($0.030 \pm 1.6 \times 10^{-3}$ $mlO_2 \cdot min^{-1} \cdot g^{-1}$) during the seven days. Surprisingly on day zero, leptin treated mice and IOP treated mice had lower mass-specific oxygen consumption rates than vehicle treated mice ($p < 0.007$ and $p < 0.012$, respectively; figure 9). Leptin treated mice also consumed more oxygen on day 7 than on day 0 ($p < 0.012$). Eating, time of day, and activity levels are all

known to affect rate of oxygen consumption. Since day zero was before any treatment, all of the oxygen consumption rates should have theoretically been similar since all of the mice exhibited similar eating and activity patterns on day zero, and the metabolic tests were done at the same time. Therefore due to the variability observed, these data may not be accurate.

The ratio of CO_2 production to O_2 consumption (RQ) changes based on the substrate being oxidized and thus may allow for a better understanding of lipid and glucose trafficking. The RQ for the leptin treated mice decreased over the seven day treatment period ($p < 0.015$), while the RQ of other groups did not show any change on a day- to-day basis (data not shown). On day 7, the leptin and IOP treated mice had lower RQ values compared to day zero ($p < 0.016$ and $p < 0.048$, respectively; figure 10). No other groups had significantly different RQ values between day 0 and day 7. Compared to vehicle treated mice on day 7, leptin/IOP treated mice had a lower RQ indicative of lipid metabolism (figure 10). It is not known if this was a treatment affect. The lower RQ may have been due to the fact that animals from this group did not eat so their only source of energy would have been reserves in adipose

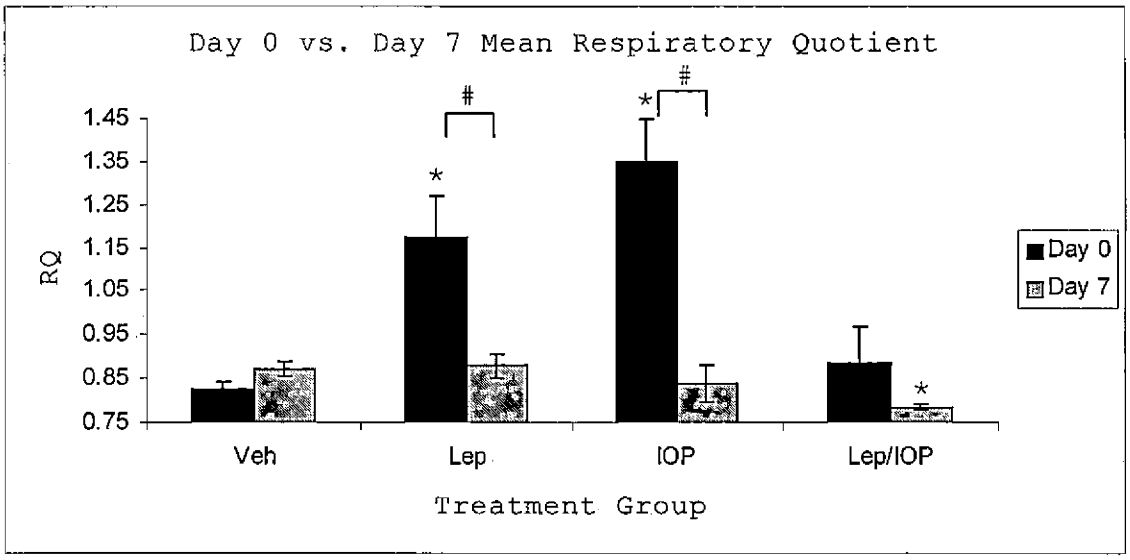


Figure 10. Respiratory Quotient (RQ) on Days 0 and 7 of the Treatment Period. On day 0, IOP treated mice had an RQ higher than the vehicle treated mice. On day 7, vehicle, leptin, and leptin/IOP treated mice had an RQ of about 0.85. This suggests that the mice were utilizing a mixture of carbohydrates and lipids to fuel metabolism. Leptin/IOP treated mice had an RQ of 0.79 after the treatment period, suggesting these mice were utilizing mostly lipids in metabolism.
*Significant difference on day 0 for vehicle vs. lep and vehicle vs. IOP, and day 7 for vehicle vs. leptin/IOP. Also significant on day 7 was leptin vs. leptin/IOP.
#Significance on day 0 vs. day 7, $p < 0.05$.
Values are mean ±SEM. For vehicle and leptin, n=5. For IOP and leptin/IOP, n=3.

stores. The leptin and IOP treated mice had RQ values similar to that of the vehicle treated mice after seven days, all of which were indicative of a mixture of both carbohydrate and lipid metabolism. As with oxygen consumption, day zero RQ was highly variable (1.05 ± 0.12)

among the mice even though this was pre-treatment. Therefore, experiments on RQ need to be repeated to understand the implications of RQ from these drug treatments.

Adipose Tissue

Omental adipose tissue is found near the stomach and spleen and can extend into the rest of the abdominal cavity. Adipocyte sizes fluctuate depending on nutrient availability. Relative to vehicle treated mice, leptin treated mice had larger adipocyte areas ($p < 0.05$), leptin/IOP treated mice had smaller adipocyte areas ($p < 0.05$), whereas the IOP treated mice were not different from vehicle treated mice (figure 11A, 12). It is not known whether the decrease in adipocyte area for the leptin/IOP treated mice was due to reduced food consumption or to the treatment. The leptin/IOP treated mice would have been expected to have adipocyte areas in between those of leptin and IOP treated mice, not lower, suggesting that the results seen for this group are due to starvation. During starvation, lipids stored in adipocytes are metabolized to support whole-body energy consumption.

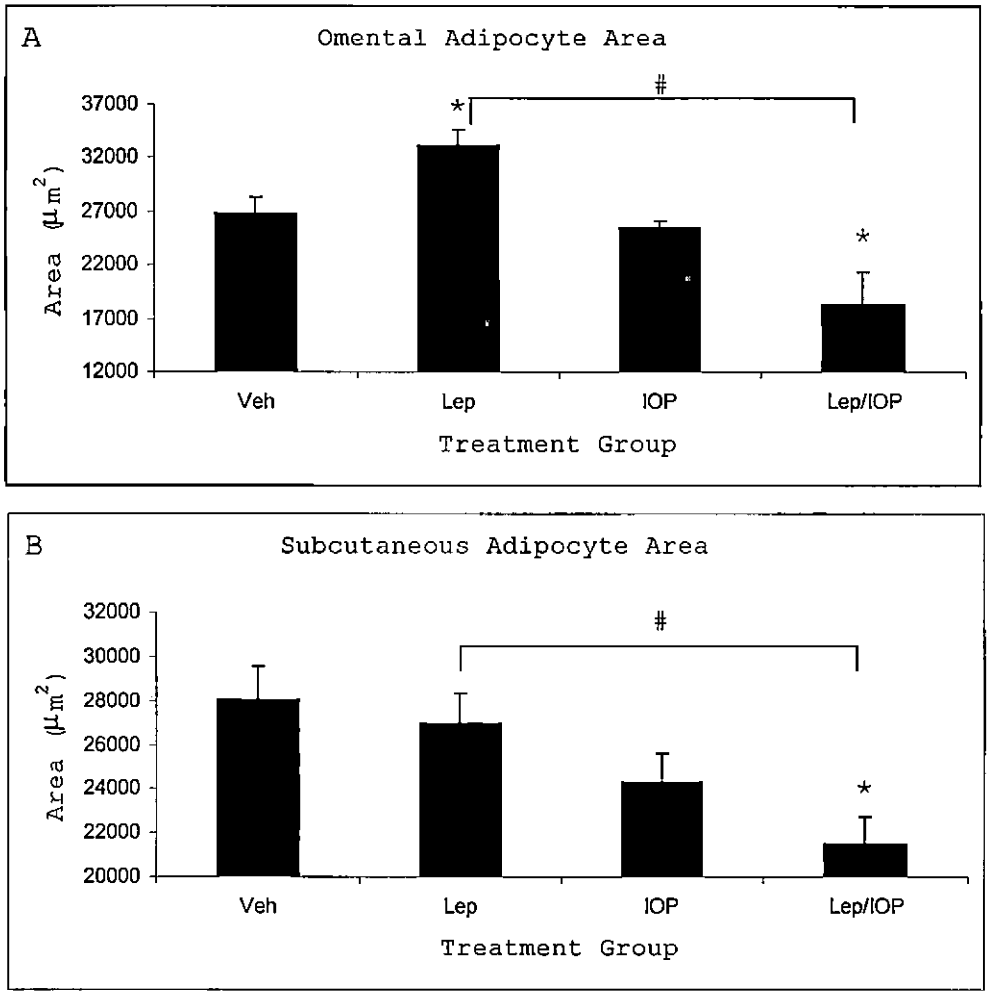


Figure 11. Omental and Subcutaneous Adipocyte Area. A. Omental adipocyte area among the different treatment groups. Relative to vehicle treated mice, leptin treated mice had larger adipocyte areas, IOP treated mice had similar adipocyte areas, and leptin/IOP treated mice had smaller adipocyte areas. B. Subcutaneous adipocyte area among treatment groups. Subcutaneous adipocyte area in the leptin/IOP group decreased compared to the vehicle group. Leptin and IOP treated mice had cell sizes similar to that of vehicle treated mice. *Significant differences relative to vehicle treated mice. #Also significantly different were in A: leptin vs. leptin/IOP; in B: leptin vs. leptin/IOP, $p < 0.05$. Values are mean ±SEM. For vehicle and leptin, n=5. For IOP and leptin/IOP, n=3.

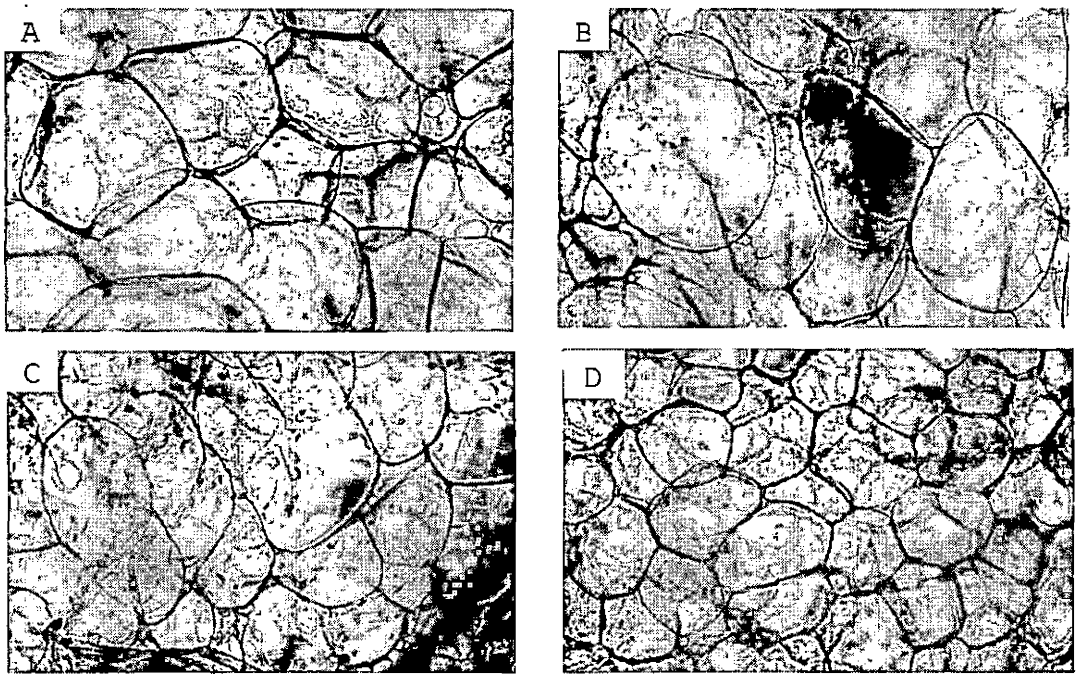


Figure 12. Omental Adipose Tissue Viewed Under the Light Microscope.
A: vehicle treated mice; B: leptin treated mice; C: IOP treated mice; D: leptin/IOP treated mice. Leptin treated mice had the largest adipocyte area, leptin/IOP treated mice had the smallest, and IOP treated mice had similar adipocyte area relative to vehicle treated mice.
Ten different fields of view were taken per animal at 200x.

Subcutaneous adipose tissue is the fat storing tissue found just beneath the surface of the skin. This fat is mainly used for insulation and long-term storage of lipid. Subcutaneous adipose stores are the primary source for lipids utilized during starvation; however, omental adipose stores can still be used (Wajchenberg 2000). However, there

were no significant correlations observed between omental adipocyte area and relative weight change.

The large omental adipocytes observed in leptin treated mice may be indicative of lipid be taken away from peripheral tissues that may have exhibited excess fat before treatment (because they are obese), but with leptin treatment they are able to redirect the lipid to omental adipose tissue for temporary storage. IOP treatment had no effect on the average area of individual adipocytes, suggesting that low thyroid hormone does not affect lipid storage in omental fat.

The leptin/IOP treated mice had the smallest area per cell in subcutaneous adipocytes; these mice had significantly smaller cells than both the vehicle and leptin treated mice ($p < 0.05$, figure 11B, 13D). The area of subcutaneous adipocytes in IOP treated mice was not different from either vehicle or leptin treated mice (figure 13).

The subcutaneous adipocytes in mice in the leptin/IOP group may have been acting as the primary energy source for these mice because they did not eat during the treatment period. This idea could be supported by a positive correlation between mean daily food consumption

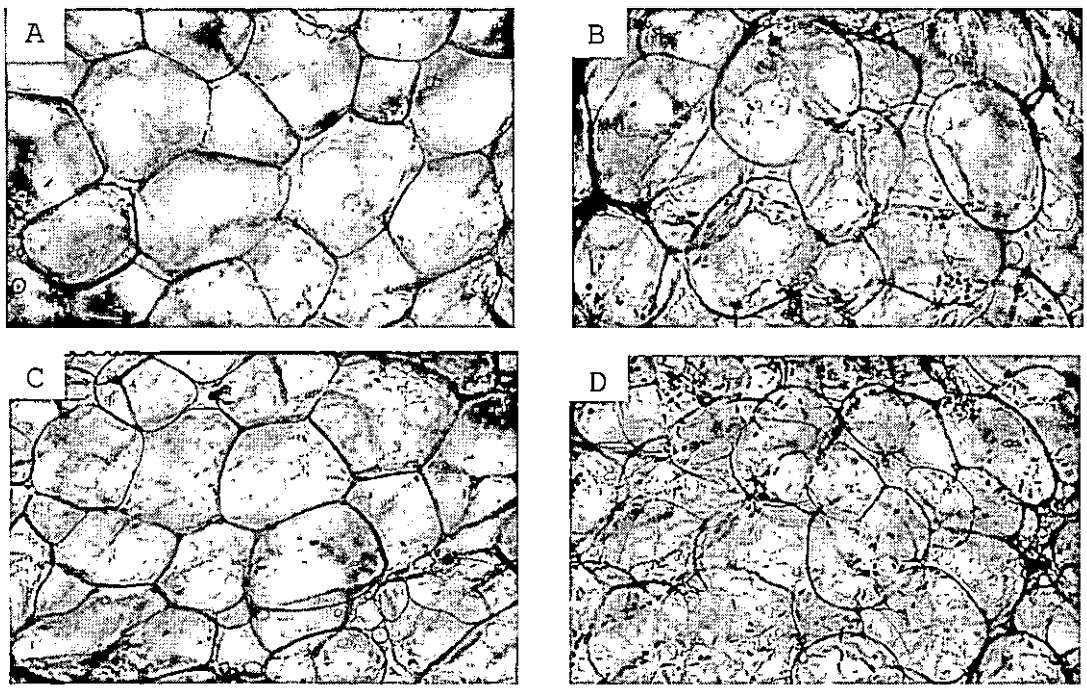


Figure 13. Subcutaneous Adipose Tissue Viewed Under the Light Microscope.
A: vehicle treated mice; B: leptin treated mice; C: IOP treated mice; D: leptin/IOP treated mice. Leptin and IOP treated mice had similar adipocyte areas to vehicle treated mice, while leptin/IOP treated mice had a smaller adipocyte area.
Ten pictures were taken per animal at 200x.

(g food/day/g Mb) and subcutaneous adipocyte areas in the leptin/IOP group; however this relationship turned out to be insignificant ($p = 0.093$, $r^2 = 0.998$, $F = 416$) because food consumption was about zero for these mice. These two variables were not correlated in any other group.

The average area of subcutaneous adipocytes in the leptin and IOP treated mice did not change relative to the vehicle mice. It was hypothesized that IOP treated mice

would show an increase in the subcutaneous adipocyte area because low levels of thyroid hormone reduce lipolysis (Oppenheimer 1991; Pou 1989). Since the mice in this group were sick at the beginning of the study, the area of the adipocytes may have decreased as these cells were providing lipids for energy. As these mice started eating again, the cell area increased. The mice treated with leptin were expected to have a decrease in subcutaneous adipocyte area as leptin acts to stimulate lipolysis and induce weight loss in obese mice (Halaas et al. 1995; Maffei et al. 1995). The results support the idea that subcutaneous lipid is primarily metabolized when energy supply is low. Since food was available and mice in the vehicle, leptin, and IOP groups all ate, their subcutaneous adipocyte area remained the same.

Hepatic Lipids

The liver stores lipids in small intracellular droplets. Both the size of the droplet and the total amount of lipid in the cell vary with metabolic demand. Total hepatic lipid droplet (LD) content was determined by calculating the cross-section area of LD in a cell (figure 14). It was seen that leptin treated mice had the least amount of lipid while leptin/IOP treated mice had the most

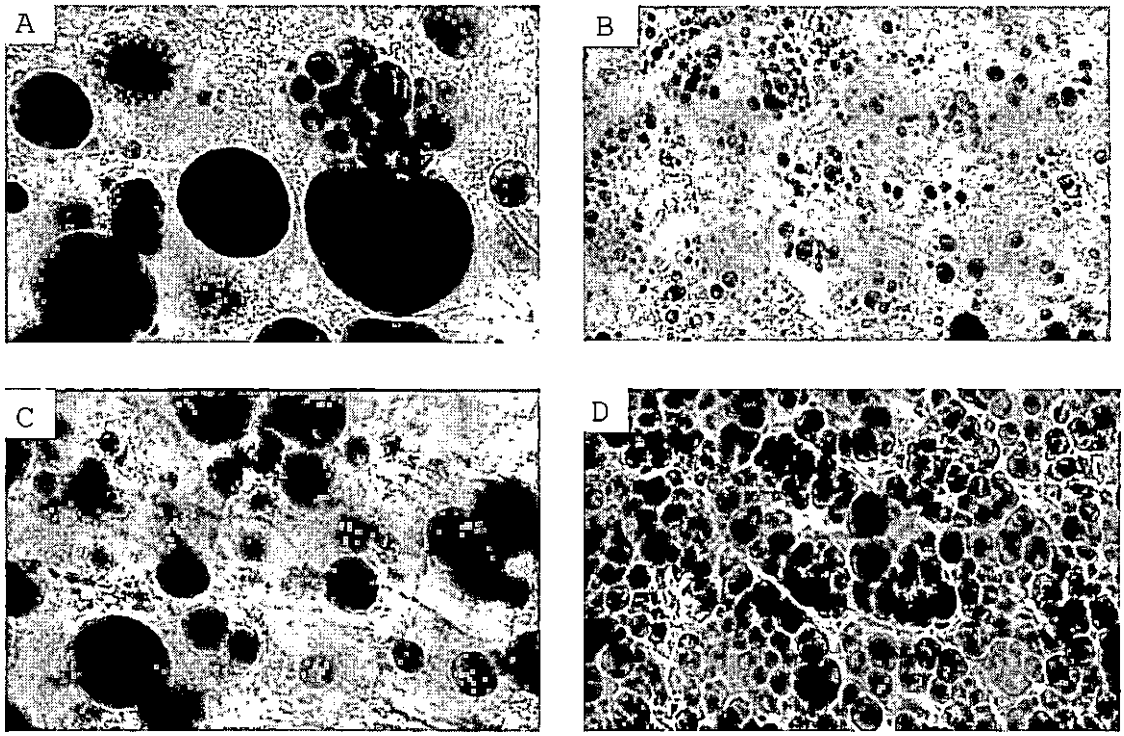


Figure 14. Hepatic Lipids Viewed Under the Light Microscope.
A: vehicle treatment; B: leptin treatment; C: IOP treatment; D: leptin/IOP treatment. Compared to vehicle treated mice, leptin treated mice had less, and leptin/IOP treated mice had more, hepatic lipid, while hepatic lipid in IOP treated mice was similar.
TEM embedded, osmium tetroxide post-fixed livers sectioned at 900 nm and viewed at 1000x.

($p < 0.05$; figure 15). The IOP treated mice had relatively similar lipid content as the vehicle treated mice. Moreover, hepatic cells from both the leptin treated mice and IOP treated mice contained significantly less lipid than those cells from the leptin/IOP treated mice ($p < 0.05$).

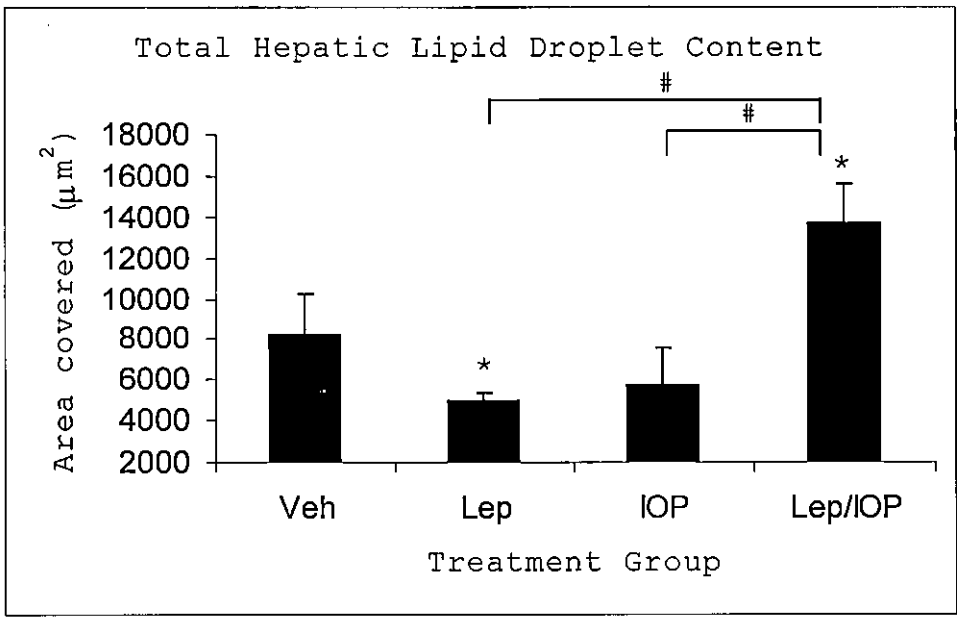


Figure 15. Total Liver Lipid Content. Compared to vehicle treated mice, leptin treated mice had less lipid droplet content, IOP-treated mice had the similar amounts of lipid droplet content, and leptin/IOP treated mice had more lipid droplet content.
*Significant differences with groups against the vehicle treated group. #Also significantly different were leptin vs. leptin/IOP, IOP vs. leptin/IOP; $p < 0.05$.
Values are mean ±SEM. For vehicle and leptin, n=5. For IOP and leptin/IOP, n=3.

I predicted that a treatment combining leptin and IOP should result in a metabolic profile similar to leptin treatment alone. The hepatocyte lipid content seen in the present study showed that leptin treatment caused a reduction in the amount of total lipid in the liver where

IOP treatment of leptin-deficient mice caused no change in lipid content, confirming previous research (Reno and Fehn 2007). The results from the leptin/IOP treatment were contradictory to expected. The total lipid content in the liver actually increased; therefore, the prediction that leptin would rescue steatosis in mice with a low thyroid hormone concentration was shown to be incorrect. This suggests that thyroid hormone may be an important factor involved in controlling hepatic lipid breakdown and/or export, as supported by Mukhopadhyay et al. (2003).

Hepatic LDs were categorized into two groups: small (< 100 μm^2) and large (> 100 μm^2), as done previously (Reno and Fehn 2007), because these are arbitrarily the two main groups of lipid droplets in the liver. All of the treatments caused a large number of small LDs in the liver, most likely because all mice are synthesizing lipid, just at various rates. Furthermore, the sizes of individual LDs within the < 100 μm^2 class were all different among the groups ($p < 0.05$; figure 16A). IOP treated mice had smaller LDs than the vehicle treated mice, while the LDs in leptin and leptin/IOP treated mice were larger than the vehicle treated mice.

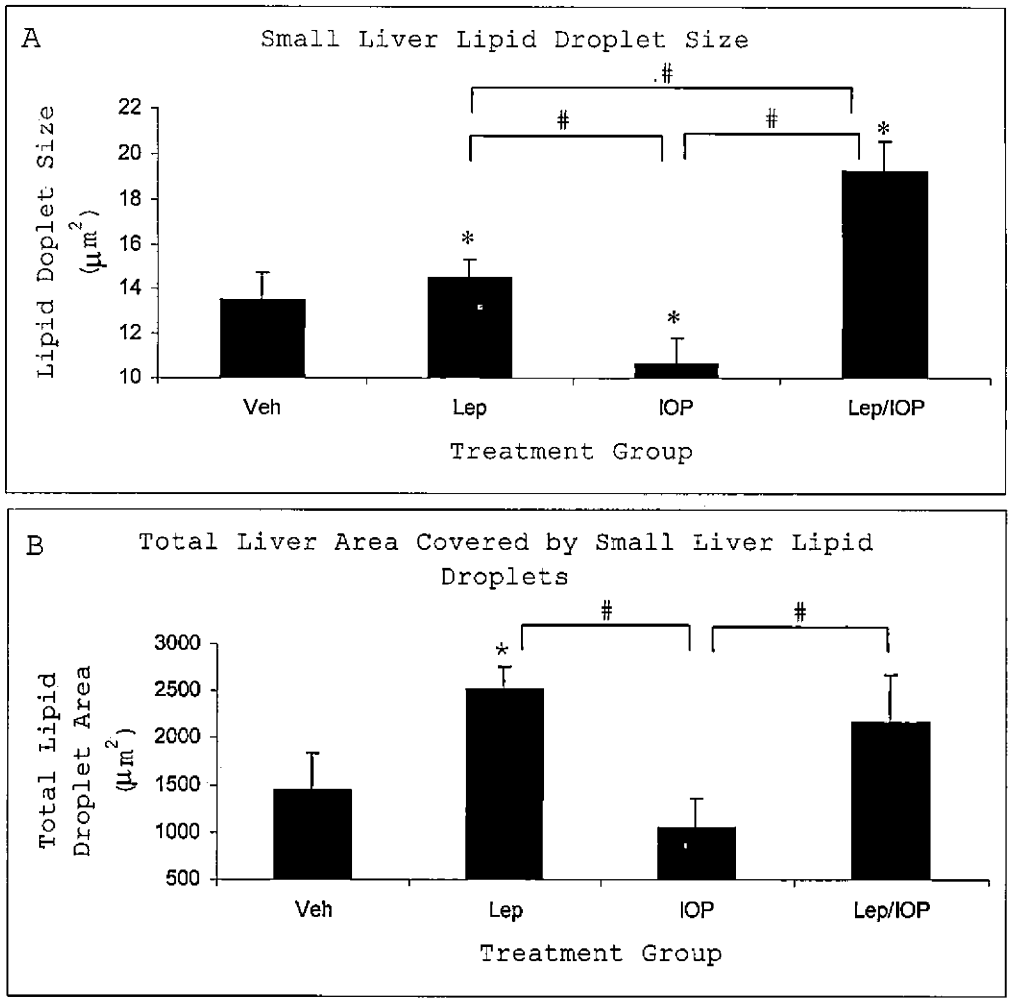


Figure 16. Small Liver Lipid Droplets.
A. Analysis of small LDs showed that leptin, IOP, and leptin/IOP treated mice had small LDs of different sizes compared to the vehicle group. Of all the small LDs, the leptin/IOP group had the largest LDs and the IOP group had the smallest. The leptin group had larger LDs than the vehicle group.
B. Total small liver LD area covered. Leptin treated mice showed more small LDs relative to vehicle treated mice. IOP and leptin/IOP treated mice had livers covered in similar amounts of small LDs to vehicle treated mice.
*Significant differences against the vehicle treated group. #Also significantly different were in A: leptin vs. IOP, leptin vs. leptin/IOP, IOP vs. leptin/IOP; in B: leptin vs. IOP, IOP vs. leptin/IOP, $p < 0.05$.
Values are mean ±SEM. For veh and lep, n=5. For IOP and lep/IOP, n=3.

The various small LDs seen within the small size category are thought to be indicative of lipid synthesis, but also could be representative of the breakdown of larger (> 100 μm^2) LDs for lipid export. Leptin treated mice were expected to have the smallest LDs of all the groups and leptin/IOP treated mice to have slightly larger LDs because the effects of low T_3 would cause less hepatic lipid breakdown, leading to larger LDs (Mukhopadhyay et al. 2003). LDs in IOP treated mice were not expected to change from those in vehicle treated mice. Even though all groups had small LDs, the total areas of a cell covered by small LD's were different. The visual analyses were supported by a nested ANOVA (figure 16B). The hepatocytes of leptin treated mice had the most area covered by small LDs while the hepatocytes covered by small LDs in IOP and leptin/IOP treated mice were not different relative to the vehicle treated mice. However, the IOP treated mice had less hepatic area covered by small LDs relative to leptin and leptin/IOP treated mice ($p < 0.0002$ and $p < 0.016$, respectively). The livers of IOP treated mice may have had smaller small LDs because their livers contain mostly large LDs (discussed below), which indicates lipid storage. While the small LDs are present in all groups, they may not be as

important in understanding hepatic lipid content as large LDs (see below).

Within the class of LD > 100 μm^2, leptin treated mice had the smallest LD (mean= 200 μm^2, $p < 0.05$; figure 17A). The large LDs in this class from the IOP and leptin/IOP treated mice were also significantly larger than those of the leptin treated mice ($p < 0.05$), but not from one another or the vehicle treated mice.

The fact that livers of leptin treated mice had the smallest LDs within the large LD class once again suggests leptin acts to decrease the size of these larger LDs in obese mice, breaking them down for export and/or β-oxidation. In leptin treated mice, small LDs (mean= 15 μm^2) predominated over large LDs (mean= 200 μm^2). It was not expected that LDs in the IOP and leptin/IOP groups would be about the same size as those of the vehicle treated mice. However, while the average size of these storage droplets may be relatively similar, the total area taken up by large LDs was different between the groups (figure 17B). The area covered by large LD in IOP treated mice was a similar amount to vehicle treated mice, but significantly greater than the leptin treated mice ($p < 0.0007$). The leptin/IOP treated mice had more area covered by large LDs than all

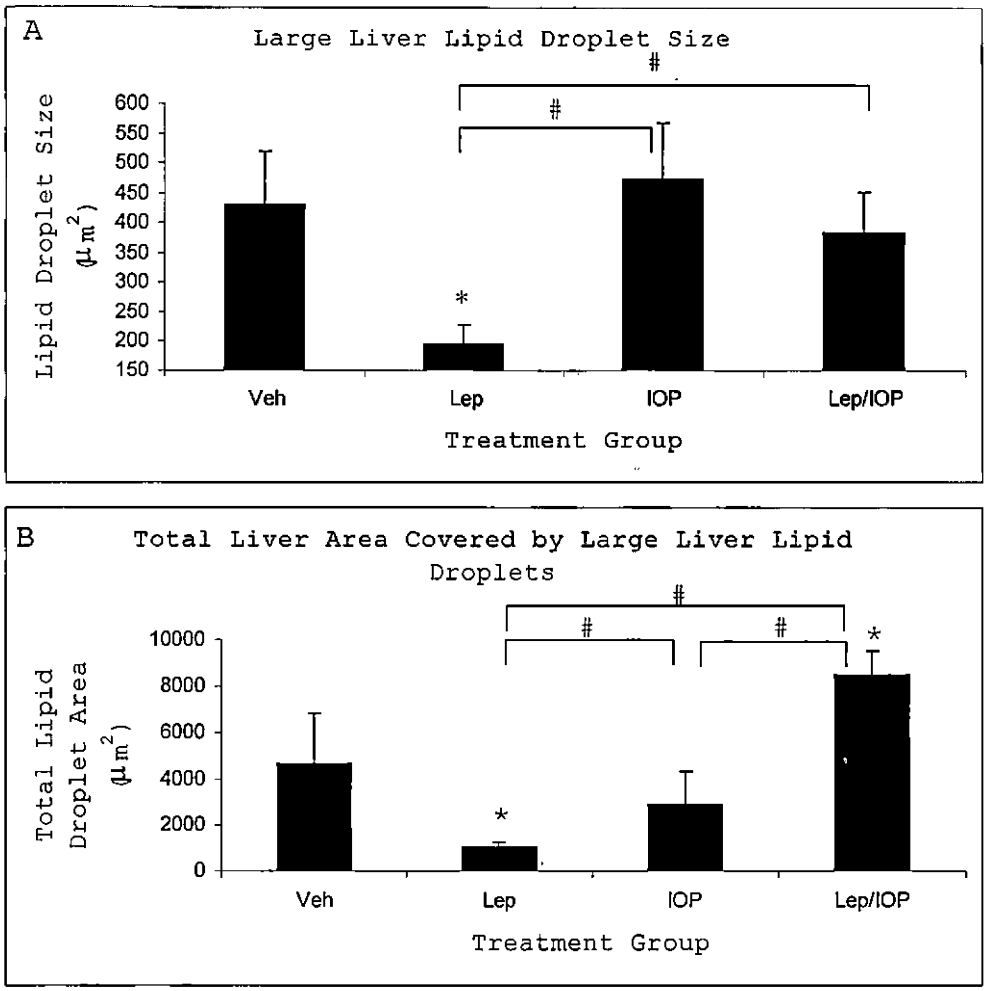


Figure 17. Large Liver Lipid Droplets.
A. Large liver lipid droplets show that the leptin group has the smallest lipid droplets in the large LD group. The IOP and leptin/IOP groups had lipid droplets not different from the vehicle group.
B. Total large liver LD area covered. Leptin treated mice had less hepatic large LDs, while leptin/IOP treated mice had more hepatic large LDs than vehicle treated mice. IOP treated mice had similar area covered in liver with large LDs relative to vehicle treated mice.
*Significant differences against the vehicle treated group.
#Also significantly different were leptin vs. IOP, leptin vs. leptin/IOP, $p < 0.05$. Values are mean ±SEM. For vehicle and leptin, n=5. For IOP and leptin/IOP, n=3.

other groups ($p < 0.00001$). The inability of leptin/IOP treated mice to break down the large LDs suggests that the action of leptin was hindered, possibly by the lack of thyroid hormone.

Taken together, the quantity and sizes of hepatic LDs suggest that while leptin treatment alone can reduce large LDs, the leptin/IOP combined treatment cannot. The greater total lipid content and the larger LDs observed in the leptin/IOP group provide more evidence for a possible leptin/thyroid hormone interaction. As mentioned earlier, previous research (Reno and Fehn 2007) suggests leptin has the ability to reverse the effects of obesity during adverse conditions, such as sympathetic neuron ablation and hyperthyroidism, whereas a low thyroid hormone environment in obese mice causes massive hepatic steatosis. Although IOP treated mice did not have more lipid or larger LDs than the vehicle group in this study, the hepatic lipid content in both of these groups is still very high (about 60-80% more lipid than a normal (*ob/+*) mouse). Since leptin replacement in conjunction with IOP did not decrease the quantity or size of the lipids as it does when given alone, the lowered T_3 levels are likely inhibiting the action of leptin. Thus thyroid hormone may be an essential factor

that works in concert with leptin to regulate hepatic lipid trafficking (i.e. T_3 may be permissive to leptin).

Conclusion

The effects of leptin and thyroid hormone on various metabolic parameters were studied in leptin-deficient obese mice. Exogenous leptin decreased liver lipid content, reduced liver lipid droplet sizes, and increased the size of omental adipose cells. All of these results confirm previous studies showing leptin has the ability to reduce excess lipid storage in the liver by shuttling lipid to adipocytes. Although the IOP and leptin/IOP treated mice became ill, this experiment still provides insight into the role of thyroid hormone in lipid trafficking. IOP treatment alone produced results similar to the control mice with regards to lipid storage in adipose tissue and liver, and the leptin and leptin/IOP treated mice showed opposite results to one another. Therefore, comparisons among the leptin, IOP, and leptin/IOP groups can still be made. The IOP treated mice were not different from the vehicle treated mice with regards to adipose tissue and liver lipid storage. Oddly, when leptin and IOP were combined, significant decreases were seen in omental and subcutaneous

adipocyte areas and increases in liver lipid content, which indicated excess lipid storage. This is surprising because my working hypothesis was that giving leptin to an obese animal in a low thyroid hormone environment should produce results that reflect a reversal of excess lipid storage that occurs in the IOP treatment alone. The mice in the combined leptin/IOP treatment actually had increased hepatic lipid content and lipid droplet size, but had the smallest adipocyte areas. The hepatic lipid should have been exported and lipid storage should have taken place in adipose tissue only. Since this occurred in the leptin treated mice and did not occur in the IOP treated mice, this suggests lack of thyroid hormone is the limiting factor regarding the ability to export hepatic lipids or to use them for β-oxidation.

Thyroid hormone is an important regulator of metabolism, and this study provides further evidence that it is essential in lipid trafficking in the liver. Further studies should be conducted that analyze thyroid hormone effects in other tissues to obtain a better picture of its role in whole-body metabolism. Further study is also needed to understand the role of thyroid hormone in the export mechanisms of hepatic lipids, especially the proteins that

may be involved in LD breakdown, export, and/or β-oxidation.

CHAPTER THREE

DISCUSSION OF CONCLUSIONS COMPARED TO PREVIOUS WORK

Discussion

In the present study, leptin-deficient obese mice were used to study how the hormone leptin acts on lipid metabolism in a low thyroid hormone environment. Metabolic parameters examined were food consumption, weight change, mass-specific oxygen consumption, respiratory quotient (RQ), omental and subcutaneous adipocyte areas, and hepatic lipid content and size.

Metabolic rate can be estimated from the rate of oxygen consumption (V_{O2}). Metabolic rate can be affected by numerous factors, including food intake, activity levels, and time of day. This study attempted to minimize variation in metabolic rate from these factors. All of the animals were allowed access to food day and night while in their cages. While in the metabolic flow-through chambers, all animals did not have access to food and their relative activity levels were noted. All mice were in the chambers for approximately eight minutes before measurements were taken to acclimate them to unfamiliar surroundings.

Furthermore, the metabolic rates were measured in the early morning hours every day. However, in spite of attempting to minimize these variations, there were large differences in pre-measurement food consumption and relative activity levels in the chambers.

Mass-specific oxygen consumption was highly variable among all twenty mice (even on day zero) before any injections were given. The RQ values were similarly variable. Due to this variation, the data from both oxygen consumption and RQ are suspect. However, all mice had a similar rate of mass-specific oxygen consumption on day seven. The RQ values on day seven were also similar (RQ = 0.86 ± 0.012) for all groups except leptin/IOP treated mice, which had a lower RQ (0.78), suggestive of metabolizing lipids. These mice had not eaten over the whole treatment period and stored lipids would have been their only source for energy. The rest of the groups had similar RQ values indicative of a mixture of lipid and carbohydrate metabolism.

Leptin is known to suppress appetite, increase metabolic rate, and cause an obese mouse to lose weight (Halaas et al. 1995; Maffei et al. 1995). The leptin treated mice in the current study did not decrease food

consumption during the treatment period compared to vehicle treated mice. However, closer examination of the data suggest that if the treatment had been continued over a longer period the leptin treated group would have exhibited significantly decreased food consumption. Unpublished work from Dr. Fehn's lab suggests that a leptin effect on food consumption becomes evident after nine days of treatment. This is supported by the difference in food consumption between vehicle and leptin treated mice shown in the trend line in figure 8. Furthermore, leptin treated mice did not decrease in body mass during the treatment. However, the vehicle treated mice did gain weight; therefore, leptin may have been acting to inhibit weight gain. Mean daily food consumption by the leptin treated mice was strongly correlated to final weight change on day seven. Leptin may have caused a reduction in food intake relative to control mice, which, in turn, reduced how much weight they gained.

Terminal analysis of omental adipose tissue and hepatic lipids showed that leptin treated mice had the largest adipocytes and the lowest hepatic lipid content. Furthermore, hepatic LDs were mostly small with the largest LDs still smaller than the other groups' large LDs. Taken together, these results suggest that leptin promoted the

breakdown of large LDs in the liver which led to exportation of the resulting small LDs to the adipocytes or utilization in β-oxidation. These results agree with those from previous work on *ob/ob* mice (Reno and Fehn 2007).

Since the IOP and leptin/IOP treated mice stopped eating during the treatment period, starvation effects must be taken into account during analysis of their data. Starvation can alter the physiological status of an animal. During starvation, glucose produced by the liver and kidneys is primarily used by the central nervous system as an energy source (Neumann-Haefelin et al. 2004). Fatty acids released from adipose stores are used for energy by peripheral tissues, such as heart, skeletal muscle, and liver. This occurs through fatty acid oxidation and through the oxidation of ketone bodies, which are by-products of excess fatty acid breakdown in the liver (Neumann-Haefelin et al. 2004). Furthermore, acute starvation is known to cause an increase in secretion of insulin, growth hormone, and glucagon leading to hypoglycemia (Goodman 1961). Starvation stimulates adipose tissue to break down triglyceride to free fatty acids (Goodman 1961). Therefore, due to the increased lipid mobilization from fat stores, the respiratory quotient (RQ) should be close to 0.7,

indicative of lipid metabolism (Von Pettenkofer 1966). During starvation, metabolic expenditure, i.e. oxygen consumption, is decreased (Therriault 1968). Therefore, the mice that stopped eating were expected to have smaller adipocytes due to starvation alone and not necessarily due to the effects of the treatments. Moreover, these mice would be expected to have a lower metabolic rate, which may cause the lipids released from adipose tissue to accumulate in other tissues.

Only the leptin treated mice showed a correlation between mean daily food consumption and body weight, as well as mean daily food consumption and the size of subcutaneous adipose cells. However, while the IOP and leptin/IOP treated mice ate less and lost a relatively large amount of body weight, there were no correlations between those variables and either of the groups. The IOP treated mice most likely did not show a relationship between the two variables because two of the three mice that remained in that group started eating and gaining weight back by day four. The one mouse that did not show this pattern may have skewed the data. An ANCOVA analysis of food consumption revealed that this one mouse was significantly different from the other two in the group.

However, due to the small sample size, this mouse was not excluded from the analyses. The leptin/IOP treated mice stopped eating all together. Thus, there would be no correlation with weight change because food consumption stayed at zero throughout the study. Therefore, the decrease in body mass in the IOP and leptin/IOP groups cannot be directly attributed to the amount of food (or lack thereof) consumed.

The leptin treated mice were expected to lose weight, while the IOP treated mice were expected to gain weight. Neither of these predicted outcomes occurred. However, the IOP treated mice which recovered showed a possible trend towards increasing body mass that may have become significant given more treatment days. Leptin treated mice were expected to lose weight. While that result was not observed, leptin treated mice did have reduced food consumption relative to the vehicle treated mice, and they also did not gain weight over the course of the study, like the vehicle treated mice did.

Despite the prediction that the cross-sectional area of adipocytes would change with changes in body weight, omental and subcutaneous adipocyte area were not correlated with day seven weight change. This suggests that these two

adipose sites may not be directly affected by acute fluctuations in body mass, but rather may have a role in long term energy balance with long term body mass fluctuations. Moreover, the duration of this study may have not been long enough to see significant effects.

The size of hepatic LD is indicative of whether the liver is undergoing lipid synthesis, disposal, or storage. Large lipid droplets (> 100 μm^2) represent a storage form of lipids, whereas small lipid droplets (< 100 μm^2) may suggest either lipid synthesis, lipid export, and/or β-oxidation. Understanding how the liver is handling lipids in response to the different treatments allows for a better understanding of the lipid trafficking that occurs during these different conditions. Small LDs were abundant in the leptin treated mice, suggesting leptin stimulates hepatic lipid breakdown by β-oxidation and/or lipid export. This is important because it leads to a reduction in fatty liver observed in *ob/ob* mice. Large LDs were abundant in the vehicle and IOP treated mice, but the livers of leptin/IOP treated mice had the most area covered by large LDs. It is not known whether starvation has a major impact on hepatic lipid trafficking; however, starvation does suppress hepatic fatty acid synthesis (Kornacker 1965). The results

seen in the leptin/IOP treated mice could be due to leptin induced lipid breakdown, but due to the high hepatic lipid content in this group, the LDs that are broken down most likely cannot be exported or undergo β-oxidation. This suggests that thyroid hormone may be required to work either in concert with leptin or independently to export lipids out of the liver or increase β-oxidation.

Comparison to Previous Work in Dr. Fehn's Lab

Leptin/thyroid hormone interactions have been seen in previous studies on *ob/ob* mice (Fehn et al. 1999; Reno and Fehn 2007). *Ob/ob* mice given exogenous leptin increased thyroid hormone concentration (Fehn et al. 1999). Moreover, leptin was previously shown to promote hepatic LD breakdown (Reno and Fehn 2007). *Ob/ob* mice given excess T_3 (thyroid hormone) in addition to leptin reduced hepatic lipid content, whereas the absence of leptin in T_3 treated mice resulted in hepatic steatosis. This suggests a possible interaction between leptin and thyroid hormone influencing lipid trafficking. Mice given IOP to reduce T_3 concentration showed massive hepatic steatosis. In the present study, IOP treatment did show hepatic steatosis, but it was to the same extent as observed in the vehicle group.

Fehn et al. (1999) found that omental and subcutaneous adipocytes were largest in leptin treated mice, and mice in all other treatment groups had similar adipocyte size. In the present study, leptin treated mice had the largest omental adipocytes, whereas leptin/IOP treated mice had the smallest areas. Vehicle, leptin, and IOP treated mice all had similar subcutaneous adipocytes while the adipocytes in the leptin/IOP group were smaller. This supports the idea that mice use subcutaneous lipid during starvation. There are no previous data on adipocyte area or hepatic lipid contents after leptin/IOP treatment.

The present studies confirm the ability of leptin to reduce hepatic lipid content and the body's ability to store lipid in the proper tissues (adipose). However, there is some kind of dysregulation in this process in the face of reduced thyroid hormone concentration. The interaction between leptin and T_3 is poorly understood and needs further investigation.

IOP and leptin/IOP treated mice decreased mean daily food consumption in the present study compared to vehicle treated mice, whereas Underhill (2000) found a decrease in only the leptin and leptin/IOP treated mice. The IOP treated mice in the present study became sick in the first

couple of days of treatment. Therefore, the differences seen within this group between the present study and Underhill (2000) may be due to the mice not eating the first couple of days. In Underhill's study, mice receiving IOP did get sick, but there is no record of them not eating. Furthermore, in Underhill's study, food consumption was not reported mass specifically.

Relative changes in body mass were different between the two studies. While both the vehicle and leptin/IOP groups in both studies showed weight gain or weight loss, respectively, the leptin treated mice in the present study did not change in body mass while the same treatment group in Underhill's study did. Moreover, IOP treated mice in the present study lost weight while in Underhill's study those mice increased in body mass. In the present study, the sickness in the IOP treated mice may be the cause of the decreased food consumption as well as the decreased body mass. I predicted that the IOP treated mice should eat similar amounts of food relative to vehicle treated mice and should gain a similar amount of weight because low thyroid hormone concentration results in decreased lipolysis and β-oxidation.

Oxygen consumption rates in Underhill's study were approximately 4 ml $O_2 \cdot min^{-1}$ for vehicle, leptin, and leptin/IOP treated mice. He did not report mass-specific V_{O2}. In the present study, all of the mice consumed about 3.00 ± 0.16 ml $O_2 \cdot min^{-1}$ (0.03 ± 0.0016 ml $O_2 \cdot min^{-1} \cdot g^{-1}$). Underhill's study showed all groups with an RQ of 0.7, indicating lipid metabolism. The RQ in the present study showed the leptin/IOP group had an RQ of 0.78, showing a tendency to utilize lipids in metabolism, but the other three groups had values of 0.86 ± 0.012, indicating both carbohydrate and lipid metabolism.

Underhill's treatments were done for nine days, whereas in the present study they were done for only seven days. This may account for some of the differences seen between these. However, Underhill also did not take body mass into account in his analyses, and mice became ill in the present study. Body weight can affect the analysis because the size of an animal will alter some of the parameters assessed. Taking all of these complicating factors into account, it is clear that metabolic parameters in the C57BL/6J *ob/ob* mice need to be reanalyzed for a better understanding of what leptin and reduced thyroid

hormone concentrations will do to lipid metabolism in the absence of starvation.

Conclusion and Future Study

Since the IOP treatment in a previous study (Reno and Fehn 2007) resulted in massive hepatic steatosis, we wondered if IOP treatment combined with leptin would have the same effect on liver lipid content as leptin treatment alone. This hypothesis was not supported. The massive hepatic steatosis observed in leptin/IOP treated mice, and lack of steatosis in leptin treated mice, may indicate that leptin and thyroid hormone work together at one of the lipid export pathways. In addition, the hepatic LD content in leptin/IOP treated mice suggests that there is obstruction in lipid breakdown as well as export. Therefore, there may be multiple pathways at which these two hormones work in concert. The hypothesis that leptin administered in combination with IOP will correct the lipid trafficking dysregulation (including reduce hepatic steatosis) observed in a lowered thyroid hormone environment was not supported. Due to the starvation that occurred in two of the groups, this study has not conclusively established a relationship between leptin and thyroid hormone in lipid trafficking in obese mice.

However, the observations and comparisons made herein further support the possibility of such an interaction. Further studies to isolate proteins involved in the process of hepatic lipid breakdown and export may reveal what such an interaction might be. Experiments that look at the role of apoB and very low density lipoproteins (VLDLs) in packing lipids for export may help us understand if this postulated interaction is at the site of hepatic lipid export. In addition, markers that follow β-oxidation may elucidate if decreased lipid metabolism in the hepatocytes is the cause of the observed steatosis. A better understanding of these processes may provide possible routes for therapy in obese patients.

APPENDIX A

LIST OF ABBREVIATIONS

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6OHDA: 6-hydroxydopanine

α-MSH: melanocortin stimulating hormone

AgRP: agouti-related peptide

ATGL: adipose triglyceride lipase

CNS: central nervous system

DAG: diacylglycerol

DIT: 3, 5-diiodotyrosine

ER: endoplasmic reticulum

HSL: hormone sensitive lipase

IOP: iopanoic acid

LD: lipid droplet

MIT: 3-monoiodotyrosine

NPY: neuropeptide Y

ob/ob: leptin deficient obese mice

POMC: proopiomelanocortin

RQ: respiratory quotient

SE: sterol ester

SNS: sympathetic nervous system

T_3: 3,5,3'-triido-$_L$-thyronine

T_4: 3,5,3',5'-tetraiodo-$_L$-thyronine

TEM: transmission electron microscopy

Tg: thyroglobulin

TG: triacylglycerol

TGH: triacylglycerol hydrolase

TR: thyroid receptor

TRE: thyroid response element

TSH: thyroid stimulating hormone

V_{O2}: oxygen consumption rate

WE: wax ester

APPENDIX B

RECIPE OF SOLUTIONS

RECIPE OF SOLUTIONS

The vehicle solution was prepared with 20 mM Tris/HCl, pH 8.0. Five mice received a daily dose of 3 µl/g Mb vehicle. Leptin (R and D3 systems, #498-OB) was dissolved in 20 mM Tris/HCl, pH 8.0, as directed by the manufacturer. Five mice received a daily dose of 3 µg/g Mb of leptin (3 µl/g Mb of 1 µg/µl). Iopanoic acid (Methimazole, Western Medical Supply) was prepared under a fume hood by pondering tablets with a mortar and pestle while the tablets were still in foil. The powder was transferred to a vial, mixed with 0.1 M NaOH, and sonicated to dissolve, giving a concentration of 15 µg/µl. This solution was combined with 0.1 M HCl and resonicated. The pH was brought to about 7.8 with 0.1 M HCl. The solution was sonicated again and diluted with distilled water. Five mice received a daily dose of 50 µg/g Mb IOP (3.33 µl/g Mb of 15 µg/µl). All of the above doses were chosen based on success with previous experimentation with the same dosages in *ob/ob* mice (Fehn et al. 1999; Reno and Fehn 2007).

The leptin and saline solutions were made, aliquoted, and stored at -20 °C until use. The IOP was made fresh every morning just prior to use.

APPENDIX C

TEM EMBEDDING

TEM EMBEDDING

The liver pieces were placed in a 2.5% glutaraldehyde/Tyrode's Cacodylic acid solution for primary fixation overnight. The next day, the tissues were washed twice with cacodylic acid buffer (ph 7.4), incubated for one hour and overnight, respectively. On day 3, the tissues were incubated with fresh cacodylic acid buffer for 30 minutes. They then were allowed to post-fix with 2% osmium tetroxide (OsO_4) for about two hours. The tissues were incubated with cacodylic acid buffer for one hour, followed by a dehydration series starting at 50% ethanol and going through to 100% dry ethanol. Each dehydration step required 45 min to 2 hours, with the higher concentrated alcohols requiring the longer incubation times. The 100% dry ethanol was replaced with 100% propylene oxide for 2 hours. This solution was then replaced with a 1:2 epon:propylene oxide mixture and allowed to incubate overnight on a rotator. On day 4, the 1:2 epon:propylene oxide solution was replaced with 1:1 epon:polypropylene oxide and allowed to incubate for 2 hours while rotating. This solution was replaced with 100% epon and allowed to penetrate into the tissues overnight while on a rotator. All the above mentioned steps were done at room temperature. On day 5, the tissues and

epon were placed into embedding capsules and allowed to polymerize for approximately 18 hours at 60° C.

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