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**ACUTE AND CHRONIC EFFECTS OF LEPTIN ON SKELETAL MUSCLE
FATTY ACID METABOLISM**

**A Thesis
Presented to
The Faculty of Graduate Studies
Of
The University of Guelph**

**by
GREGORY R. STEINBERG**

**In partial fulfillment of requirements
for the degree of
Doctor of Philosophy
June, 2002**

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ABSTRACT

ACUTE AND CHRONIC EFFECTS OF LEPTIN ON SKELETAL MUSCLE FATTY ACID METABOLISM

**Gregory Robert Steinberg
University of Guelph, 2002**

**Advisor:
Dr. D.J. Dyck**

Leptin reduces food intake and increases energy expenditure in rodents through actions on both central and peripheral tissues. In rodents, skeletal muscle is an important target of leptin and previous studies have demonstrated that leptin both acutely and chronically reduces intramuscular triacylglycerol (TG). Despite the pronounced effects of elevated leptin levels in rodents, high levels of leptin in obesity suggest the development of leptin resistance. Therefore, the objectives of this thesis were to 1) determine the mechanisms by which leptin chronically reduces intramuscular TG stores in rodents and 2) examine the development of leptin resistance in rodent and human skeletal muscle.

Chronic leptin treatment (14 days) repartitioned FA towards oxidation and away from TG storage while also enhancing TG hydrolysis as measured in isolated soleus muscle using the pulse-chase technique. Increased oxidation rates and TG hydrolysis were not due to increased oxidative capacity or increased expression of hormone sensitive lipase respectively. Despite increased FA oxidation, FA transport in skeletal muscle was reduced due to reduced FA transport protein expression (FAT/CD36 and FABPpm) at the level of the plasma membrane. Thus, a reduced rate of FA transport combined with higher rates of FA oxidation and TG hydrolysis reduced intramuscular TG, possibly resulting in improved insulin sensitivity.

Direct evidence for leptin resistance in peripheral tissues such as skeletal muscle did not previously exist. Therefore, we investigated whether leptin's acute stimulatory effects on skeletal muscle FA metabolism would be reduced following high-fat feeding. In both high-fat groups (safflower and fish oil), the stimulatory effect of leptin on muscle lipid oxidation and hydrolysis was eliminated. Partial substitution of fish oil resulted in the restoration of leptin's inhibition of TG esterification.

Based on the above experimental results, we hypothesized that during the development of human obesity skeletal muscle also becomes resistant to leptin. We tested this hypothesis by measuring FA metabolism with and without leptin, in rectus abdominus muscle strips from lean and obese women. In lean women, leptin repartitioned FA towards oxidation and away from esterification. This effect was eliminated in the obese, thus providing the first evidence of leptin resistance in obese human skeletal muscle. We conclude that while leptin increases skeletal muscle FA metabolism in rodents and lean humans, it is ineffective in rodents fed high-fat diets and obese humans.

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Chapter 1: Review of Literature

A) Leptin

i) Introduction

Nearly 50 years ago Kennedy hypothesized about the presence of a circulating, lipostatic, negative feedback signal acting centrally to alter energy expenditure and food intake (179). Parabiosis experiments (experiments in which animals are surgically joined together and share a common circulation) in the following decade confirmed the presence of such a circulating factor and identified mouse mutations that lacked the lipostatic signal (*ob/ob* mice) or caused insensitivity to the signal (*db/db* mice) (81). Despite these early insights into the regulation of body mass the understanding of this lipostatic signal remained elusive. In 1994, using positional cloning, Zhang et al. (368) identified and sequenced the *ob* gene and its protein product, leptin (from *leptos*, for thin) in the *ob/ob* mouse. These mice, which had been extensively studied for the past several decades were obese, hyperphagic, diabetic, infertile, and exhibited reduced physical activity and thermoregulation. Shortly thereafter, a cohort of studies (61, 144, 260) demonstrated that recombinant leptin injection in the *ob/ob* mice rapidly reduced body mass and percent body fat while maintaining lean muscle mass and also restoring reproductive function and euglycemia. Interestingly, the effects on insulin sensitivity and reproductive function were restored independently of food intake and before significant alterations in body composition, suggesting leptin could act directly on peripheral tissues (61, 260). Later in 1995, the leptin receptor

was cloned and identified in multiple tissues with high densities being found in the appetite controlling areas of the of the hypothalamus as well as several peripheral tissues including heart, liver, pancreas and skeletal muscle (331). In contrast to the previously mentioned leptin-deficient rodent models of obesity, Maffei et al. (213) demonstrated that in most cases obese humans have elevated levels of leptin even when corrected per kilogram fat mass, suggesting that obese humans are leptin resistant. These momentous findings started a renaissance in the study of body mass regulation which has resulted in ~5000 publications on leptin over the past seven years and has permanently altered our understanding of metabolism and the regulation of body mass.

While initially it was believed that the primary physiological role of leptin was to prevent obesity by regulating food intake and thermogenesis through actions exerted primarily in the hypothalamus, this idea was challenged by evidence demonstrating pronounced effects of leptin in regulating peripheral fatty acid (FA) metabolism. In 1997, leptin was found to reduce triacylglycerol (TG) stored in pancreas, liver and skeletal muscle independently of the central nervous system (299). Later in 1997, Muoio and colleagues (241) demonstrated that leptin acutely reduced intramuscular TG by repartitioning FA towards oxidation and away from storage in isolated rodent skeletal muscle. These findings led to a proposal that a primary function of leptin may be to prevent the accumulation of FA in non-adipose tissue as this accumulation in skeletal muscle and pancreatic islets.

common in obesity, is strongly associated with the development of insulin resistance and lipotoxicity (339-341). Therefore the primary purposes of this thesis were to examine 1) the mechanisms of chronic leptin exposure on the regulation of skeletal muscle FA metabolism in rodents, 2) the role of leptin resistance in mediating leptin's effects on skeletal muscle FA metabolism in a commonly used model of insulin resistance (high-fat feeding in rodents) and 3) leptin resistance in human obesity.

The following review will begin by first highlighting some of the important findings in the leptin literature. The regulation of skeletal muscle FA metabolism as it relates to the development of obesity and the relationship between the dysregulation of skeletal muscle FA metabolism common in obesity and the development of skeletal muscle insulin resistance will then be discussed. Lastly, the review will discuss the role of leptin in mediating skeletal muscle glucose and FA metabolism and how the development of leptin resistance in skeletal muscle could lead to the altered rates of FA metabolism observed in the obese.

ii) *Regulation of leptin expression*

Leptin is a 16 kDa cytokine-like peptide that is synthesized by the *ob* gene and secreted primarily by adipocytes, but has also been shown to be produced in muscle cells (348), gastric epithelium (17), and placenta (218). While several regulatory elements have been identified within the *ob* gene promoter (cyclic AMP, glucocorticoid response elements) the sites within the *ob* gene responsible for regulating expression have not been identified, although it may respond to changes in TG content, lipid metabolites, or mechanical factors associated with stretching of the adipocyte (5, 6). In both rodents and humans, there is a logarithmic correlation between circulating levels of serum leptin and total body fat mass, which is probably explained primarily by increased release of leptin from large compared with small fat cells (207). On average leptin release per gram of adipose tissue is two times greater in obese than in lean subjects. Because fat cell size is usually enlarged 2-4 times in the obese, when expressed per fat cell, leptin secretion is up to 7 times higher in obese than in lean subjects (124). In addition, an increased number of fat cells particularly in extreme obesity undoubtedly contribute to increases in serum leptin (124). Although it was assumed that leptin was secreted immediately upon transcription due to the relation between leptin mRNA and leptin secretion (207) a recent report indicates that adipose tissue contains a significant store of leptin (~ 3 h) within membrane bound fractions (282) which may help explain the pulsatile and periodicity of leptin production (306, 307). The kidney has been found to be an important site of leptin clearance

as total nephrectomy in rats (91, 92, 231) and chronic renal failure in humans (172) results in increased plasma leptin concentrations. Despite the importance of the kidney, increased leptin levels observed in the obese are not believed to be due to reduced leptin clearance in the kidney but instead are believed to be the result of increased leptin production from adipose tissue (231).

Plasma leptin levels increase with weight gain and decrease with weight loss, consistent with leptin's role as a signal of adipose tissue stores (154, 213). Leptin levels in humans also display a diurnal rhythm with the highest levels between 1100 and 0100h, after which plasma leptin declines until early afternoon (286, 306). This diurnal rhythm is linked to meal timing because a 6-h delay in meals produces a similar phase shift in the plasma leptin profile (286). Therefore, while the long-term regulation of leptin synthesis and secretion is related primarily to the degree of adiposity, circulating leptin levels are also subject to nutritional regulation (82). While a detailed description of all factors capable of mediating leptin production is not within the scope of this review for a brief outline please see Table 1.

Table 1. Regulation of leptin expression

Site	Increase	Decrease
Adipose Tissue	Overfeeding Obesity (except <i>ob</i> mutation) Glucocorticoids Acute infection Cytokines (TNF- α , IL1)	Fasting Testosterone Beta-adrenergic Thiazolidinediones Thyroid hormone Cold exposure n-3 PUFA
Placenta	Insulin Glucocorticoids Hypoxia	Smoking Low Fetal Weight
Skeletal muscle (rat)	Glucosamine Glucose Lipids	
Stomach fundus		Feeding Cholecystokinin

Of potential interest in the context of this thesis is the regulation of leptin by insulin. (94, 154, 190, 192, 217). In rodents, insulin stimulates leptin expression directly in isolated adipocytes and also increases leptin levels when injected *in vivo*. In contrast, leptin is decreased in low insulin states, such as streptozotocin-induced diabetes but can be restored to normal levels with insulin treatment (24). Corresponding with changes in insulin, leptin levels increase within hours (4-7 hrs) after a meal while fasting induces a fall in the leptin levels which is rapidly reversed with refeeding (24, 40, 191). While early studies in humans examining the relationship between insulin and leptin were equivocal, more recent reports, which take into account changes in diurnal variation (286), and a more delayed

response compared to rodents indicate, that hyperinsulinemia increases plasma leptin levels when compared with those of saline-infused controls. *In vitro* studies also support a direct role for insulin in regulating leptin production as human, cultured adipose tissue fragments incubated with insulin increased leptin release by 50% (124). Serum leptin declines gradually during 24-36 h of fasting to 40-70% of baseline in both lean and obese subjects without an appreciable loss of fat mass (40, 190, 270), while acute massive overfeeding increases plasma leptin by 40% after 5 h (7, 193). Also highlighting the potential role of insulin in regulating leptin production is the finding that compared with a high fat diet (60% kcal), a low fat (20%), high carbohydrate diet (resulting in a greater insulin spike) produces higher peaks of leptin during the night (154, 208).

Genetic mutations in rodents and humans. In *ob/ob* mice mutations due to a Cys- to Thr substitution which results in a stop codon at position 105 leads to the production of a truncated protein that is incapable of being secreted. This results in a phenotype characterized by leptin deficiency, hyperphagia, hypothermia, insulin resistance, reproductive dysfunction and early onset morbid obesity (368). Human *ob* gene mutations are rare and were first reported in two children from a Pakistani family in 1997 (114). In these patients, deletion of a single guanine nucleotide in codon 133 led to a frameshift mutation and synthesis of a truncated leptin protein. A second mutation similar to that seen in the *ob/ob* mouse has recently been found in three members of a Turkish family (255). As with the *ob*

mutation described above, the abnormal leptin protein is incapable of being secreted and patients exhibit hyperphagia, morbid obesity, amenorrhea and hypothalamic hypogonadism (255). Physiologic replacement for one year with recombinant leptin leads to substantial weight loss and the reversal of the physiological abnormalities (255).

iii) The Leptin Receptors

The leptin receptor (Ob-R) was first isolated from mouse choroid plexus by expression cloning (331) and is a member of the interleukin-6 receptor family of class I cytokine receptors (331). Positional cloning of the OB-R gene, showed that this gene encodes five alternatively spliced forms of the leptin receptor: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re (201, 331). Ob-Rb is the long form of the leptin receptor (also known as ObR-L) and has a long cytoplasmic region containing several motifs required for signal transduction (discussed below) (332). ObR-L is found in high concentrations in the brain and more specifically in areas which regulate feeding such as the arcuate, dorsomedial and ventromedial hypothalamic nuclei (332). It is also found in low concentrations (5-8% of total Ob-R) in several peripheral tissues including adipose tissue, ovaries, testis, placenta, adrenal medulla, liver, pancreatic beta-cells, lung, jejunum, peripheral blood mononuclear cells, articular chondrocytes, heart and skeletal muscle (56, 62, 118, 221, 287, 332). In contrast, the short forms of the receptor ObR-S (Ob-Ra, Rc, Rd, Re) are present at very low concentrations in the hypothalamus but are ubiquitously expressed within the microvessels and choroid plexus of the brain as

well as all the peripheral tissues listed previously (66, 331, 332). Based on the ability of the brain microvessels to bind and internalize leptin (134), and the abundant expression of ObR-S it has been suggested that leptin may cross the blood brain barrier by transcytosis mediated by the short form of the leptin receptor. While the reason that several distinct short intracellular domain forms of ObR-S are produced is not clear, the different short forms clearly have distinct tissue distributions. However, it is currently unknown whether they have alternate functions.

Genetic mutations in rodents and humans. Leptin receptor mutations cause early onset obesity in rodents (66, 331, 332). In *db/db* mice, which are phenotypically identical to *ob/ob* mice, a premature stop codon inserted in the 3'-end of the ObR-L mRNA transcript leads to the synthesis of a truncated receptor that replaces the ObR-L isoform with the Ob-Ra isoform, which is incapable of fully mediating JAK-STAT signaling (to be discussed in greater detail below) (36, 66, 332). Ob-Ra and other ObR-S splice variants are expressed normally in *db/db* mice (66, 201). In the *fa/fa* or Obese Zucker rat, a Gln-to-Pro substitution at amino acid position 269 in the extracellular domain decreases cell surface ObR-L expression and reduces the signaling capacity of the receptor (72, 73). In humans ObR-L mutations are extremely rare but in three sisters from a Kabilian family a homozygous mutation in the human leptin receptor gene (Guanine to Adenine substitution) resulted in a truncated leptin receptor lacking both transmembrane and intracellular domains (76). This data demonstrates that the long isoform of the

leptin receptor is essential for mediating leptin's effects, as the *db/db* mouse, *fa/fa* rat and humans with ObR-L mutations are all phenotypically identical to the leptin-deficient state seen in *ob/ob* mice.

Of much greater biological significance is the recent finding by three studies that have identified several leptin receptor single nucleotide gene polymorphisms that in turn have been shown to result in increases in BMI, fat mass, serum leptin levels and impaired insulin sensitivity in humans (271, 355, 356). These studies suggest that polymorphic variations in the leptin receptor gene may be associated with variation in leptin binding resulting in differences in leptin levels, fat mass and insulin sensitivity. Clearly more research is needed to identify the prevalence of these single nucleotide polymorphisms within the population and if highly prevalent, develop new tailored therapeutic interventions for the variant form of the leptin receptor. These genetic polymorphisms may provide a significant genetic background for the development of leptin resistance and obesity.

iv) Leptin Signaling

The leptin receptor is a member of the class I cytokine receptor superfamily (332). Thus, similar to other receptors of this class, Ob-R lacks intrinsic tyrosine kinase activity and therefore requires recruitment of the activated receptor-associated kinases of the Janus family (JAKs) (18). After ligand binding, JAKs autophosphorylate and tyrosine phosphorylate various STATs (signal transducers

and activators of transcription). Activated STATs then dimerize and translocate to the nucleus, where specific gene responses are elicited (130). Both the long and short forms of the leptin receptor have the ability to activate JAK2, IRS-1 tyrosine phosphorylation and the activation of MAPK in a leptin dose dependent manner (36) although it is clear that the ObR-L more robustly activates (~5 fold greater) these pathways than does ObR-S. In contrast, only ObR-L is capable of activating STAT3 tyrosine phosphorylation and *c-fos* gene transcription (36). In peripheral locations such as articular chondrocytes, insulinoma cells, peripheral blood mononuclear cells, liver, the small intestine, white adipose tissue, and myotubes (55, 56, 111, 118, 175, 239, 287, 354) which contain predominately short forms of the leptin receptor, leptin is still capable of activating both STAT-1 and STAT-3 suggesting that the small amounts of ObR-L present in peripheral tissues is mediating leptin's effects (187). Additional evidence for this statement can be drawn from the totally leptin insensitive and obese *db/db* mice which lack only the ObR-L isoform and are unable to activate STAT despite having normal or near normal amounts of ObR-S (66, 201). Therefore, what is the role of the short form of the leptin receptor, which is expressed ubiquitously at such high concentrations? Two primary possibilities are worthy of note. First, leptin signaling may require combined input from STAT, IRS-1, and MAPK pathways, but such actions of ObR-S are not seen in the absence of the STAT input when ObR-L is not present. Secondly, the weak ObR-S signaling may subserve their primary function, which may be to increase receptor mediated ligand

internalization and binding therefore leading to greater leptin signaling by ObR-L. Clearly, more research needs to be completed to fully understand the interaction between short and long forms of the leptin receptor and the importance each plays in mediating leptin's effects.

An alternative leptin signaling pathway involves activation of the insulin signaling PI-3 kinase pathway in myotubes, insulinoma cells, hepatocytes and soleus muscle *in vitro* (153, 175, 242, 370) as well as liver, adipose and gastrocnemius muscle *in vivo* (187). Specifically, *in vivo* injection of leptin results in a small activation of IRS-1-associated PI3-kinase activity in adipose tissue and IRS-2-associated PI-3-kinase activity in liver and no activation of Akt in adipose tissue, muscle or liver compared with the large effect of insulin on these signaling steps (187). The effects of leptin and insulin on MAPK activation are additive suggesting that these two hormones affect MAPK via distinct upstream signals (187) suggesting the convergence of both the STAT and PI-3 kinase pathways at or before MAPK. Lastly, the kinetics of the effects of leptin and insulin also appear to differ as leptin results in a slower but more sustained activation of MAPK when compared to the fast acting and quickly waning effects of insulin (187). Taken together, these observations indicate that while leptin and insulin activate some of the same intracellular signaling events, the pathways also diverge indicating the presence of distinct pathways from insulin and suggesting a limited capacity for leptin to stimulate glucose transport and glycogen synthesis in skeletal muscle.

v) Leptin Action in the Brain

Leptin is transported into the rodent brain by a saturable process believed to be mediated by short leptin receptor isoforms which are highly expressed in brain microvessels (20, 189) and reduced transport of leptin across the blood brain barrier may be a potential site of leptin resistance in the obese (to be discussed in detail below). As stated previously, the highest concentration of the long form of the leptin receptor is localized within the appetite controlling centres of the hypothalamus (109, 216, 297). Hypothalamic neuropeptides involved in leptin action may be classified into two major groups: 1) those that stimulate feeding (orexigenic peptides) and are inhibited by leptin and whose mRNAs increase in response to leptin deficiency and 2) those that decrease feeding (anorexigenic peptides) that are stimulated by leptin and tend to decrease in response to leptin deficiency. Notable orexigenic peptides include neuropeptide Y (NPY), agouti-regulated peptide (AgRP), melanin concentrating hormone (MCH), and orexin. Those that decrease feeding are α -melanocyte stimulating hormone (α -MSH, product of proopiomelanocortin-POMC), cocaine-and amphetamine-regulated transcript (CART) and cotropin releasing hormone (CRH) (5, 7). The vast array of appetite controlling peptides demonstrates the significant complexity and large degree of redundancy in the regulation of energy intake suggesting that it will take years of intensive research to fully understand the leptin mediated neuronal networks.

While numerous studies have demonstrated the potent role of leptin in inhibiting food intake in rodents, initial studies in humans also suggest a potentially important role of leptin in regulating food intake in humans. For instance, significant correlations have been plotted between the quantity of habitual food intake and circulating leptin levels (198). In addition recent clinical trials have demonstrated reduced appetite and hunger as well as self reported daily caloric intake in obese patients with elevated leptin levels (159, 360) and leptin deficient patients (253, 255) injected with recombinant leptin.

vi) The Adipoinular-Axis

Leptin eliminates hyperinsulinemia in *ob/ob* mice independently of food intake and without the loss of appreciable body mass (260) suggesting that leptin may have an inhibitory role on insulin release from the pancreas. Several recent studies *in vivo* have also found that leptin treatment in non-obese rodents via injection, osmotic mini-pump, or gene therapy (insertion of recombinant adenovirus containing leptin cDNA) lowers plasma insulin levels (21, 22, 70, 107, 351). The discovery of functional ObR-L in pancreatic islets provided further evidence for the possibility of leptin involvement in insulin secretion (63). Additional evidence of leptin's effects on insulin secretion has been demonstrated in isolated pancreatic islets and perfused pancreas isolated from *ob/ob* mice which after being exposed to leptin (6.25-100 nM) for 1-2 h reduce insulin secretion from ~13 to

80% (181). Leptin has also been shown to inhibit insulin production in normal rodents and cultured human islets to a similar extent (181). Leptin attenuates insulin release in islets stimulated by either acetylcholine or the phorbol ester phorbol-12-myristate 13-acetate (PMA), both of which are activators of protein kinase C (PKC) (8). Leptin also has been demonstrated to suppress cAMP levels through a phosphoinositol 3-kinase (PI-3-kinase)-dependent activation of cyclic nucleotide phosphodiesterase 3B, thereby preventing insulin release (8, 369, 370).

This effect is blocked by application of the PI 3-kinase inhibitors wortmannin and LY-294002 (153). Therefore, leptin antagonizes insulin secretion from β -cells by interacting with both cAMP-dependent protein kinase A (PKA) and PKC signaling pathways which in turn will result in activation of K_{ATP} channels resulting in a drop in cytosolic calcium and thereby preventing insulin release (153). Similar results have also been reported *in vivo* (367). These findings (in combination with the role of insulin in increasing leptin production in adipocytes as discussed previously) highlight both the interactions and synergy between leptin and insulin signaling which represent a significant complication associated with studying these hormones *in vivo*.

B) Skeletal Muscle Fatty Acid Metabolism in Obesity

i) Introduction

Obesity is defined medically as a state of increased body weight, more specifically adipose tissue, of sufficient magnitude to produce adverse health consequences (11). The World Health Organization defines obesity as an individual with a body mass index (BMI) of $>30 \text{ kg/m}^2$ (calculated as weight in kilograms divided by the square of height in meters). The list of health consequences associated with obesity is extensive ranging from cardiovascular disease, hypertension, osteoarthritis, certain types of cancer and type 2 diabetes mellitus (245). Recent data demonstrate that greater than 50% of North American adults are overweight with a BMI of more than 25 kg/m^2 (235). This represents an increase of 50% in the past 20 years (235). Of even greater concern is the finding that the number of overweight children has more than doubled in the past eight years presaging even greater medical harm in decades to come (235). What has accounted for this epidemic of increased energy storage? Body weight and composition, and the storage of energy as TG in adipose tissue are determined by the interaction between genetic, environmental, and psychosocial factors (310). These influences ultimately act by changing the energy balance equation, that is the long-term balance between energy intake and expenditure. Daily energy expenditure consists of resting energy expenditure, the energy required to metabolize food (thermic effect of food), and energy expended as a result of activity (209). When energy intake and expenditure are in balance, body mass remains stable while a

net excess in energy, whether through greater intake or less expenditure leads to weight gain (209).

Habitability studies suggest that as much as 70% of the variability in human body mass may be accounted for by genetic factors (263), yet it is impossible that changes in the genetic background of Western society over the past 2 decades are responsible for the trend of increasing body mass. Instead it has been suggested that the predisposition for obesity is the result of a “thrifty” genotype (246), which maximizes our ability to store fat in times of nutritional abundance in order to survive through intermittent periods of famine. This positive trait, which has been selected over many thousands of years of human evolution, has only recently emerged as a harmful factor in Western society, due to large-scale changes in the environment as a result of easy access to high-energy density foods combined with a decrease in physical activity.

An obese human of approximately 115 kg has sufficient adipose stores to survive a total fast of approximately 150 days (310)! The primary tissue capable of utilizing this massive energy reserve is skeletal muscle, which by virtue of its mass (~30% of whole body mass in obese humans) is the major tissue contributing to basal metabolic rate and whole body glucose and lipid oxidation at rest and during exercise (377). Therefore, alterations in the regulation of skeletal muscle metabolic rate and fuel utilization play an important role in energy expenditure.

Skeletal muscle of the obese has increased lipid content as reported on the basis of biochemical extraction of lipid from biopsies of vastus lateralis muscle (256), histological staining with Oil Red O (139, 156, 214, 215, 266), and observations with electron microscopy (202) and by several non-invasive imaging methods, including computed tomography (138, 283), and magnetic resonance spectroscopy (16, 265, 347), this last method offering the potential to identify and quantify intracellular and extracellular content of lipid. Although the lipid content in skeletal muscle is quite small (~11, 000 kJ) relative to that in adipose tissue (343), it is still of biological significance as it is strongly associated with insulin resistance (discussed in detail below) (16, 138, 139, 156, 176-178, 202, 256, 266, 347). The mechanisms that cause skeletal muscle lipid content to increase in obesity are unclear, but might be broadly postulated to be due to i) an increased uptake of FA, ii) decreased FA oxidation, iii) increased FA esterification, iv) reduced TG hydrolysis or v) some combination of all these processes. Therefore, the primary objective of this section will be to examine the regulation of skeletal muscle lipid metabolism as it relates to obesity.

ii) Long Chain Fatty Acid Transport

The first step in the regulation of FA metabolism involves the movement of FA across the sarcolemma. While the role of plasma membrane transporters in mediating the uptake of glucose (140, 276) and lactate (41) is well documented, it was initially believed that FA due to their hydrophobic nature were capable of passively diffusing across plasma membranes. This belief was reinforced by observations of rapid FA diffusion ($t_{1/2} = 20 \text{ msec} - 2 \text{ sec}$) in purified protein-free phospholipid bilayers, suggesting that a specific transport system was unnecessary (223, 376). More recently this dogma has been challenged in light of several structural and physiological considerations. First, due to their low solubility in aqueous media and their ionized charge, 99.9% of FA circulate in plasma, bound to high affinity binding sites on the soluble protein albumin (336). According to conventional theories on cellular uptake, only an unbound ligand participates in the uptake process, which in plasma represents only 0.1%, a concentration far too low to explain the high cellular influx rates observed *in vivo* in skeletal muscle. Therefore, in order for passive diffusion to occur at rates observed *in vivo* FA would need to dissociate from albumin, which seems highly unlikely when considering the high affinity of albumin for FA (10^8 M^{-1}) (336). Lastly, at physiological pH, FA exist in plasma as anions and thus have to be taken up against an unfavorable electrical gradient due to the negative charge on the cytosolic side of the plasma membrane which hinders ($t_{1/2} = \text{min}$) their unassisted diffusion across the charged membrane (223). Therefore, it is highly unlikely that

the disassociation of FA from albumin, and their absorption and passage across the plasma membrane can occur via passive diffusion alone at high enough rates to sustain skeletal muscle at rest and during exercise.

More recent data suggests that instead FA traverse the plasma membrane via both passive diffusion and a protein-mediated mechanism (46). This suggestion is based on a number of recent findings. First is the observed saturation kinetics typical of a transport mediated process observed in both cardiac myocytes as well as giant sarcolemmal vesicles derived from red and white skeletal muscle (46, 212). Secondly the ability of heat denaturation, trypsin treatment, phloretin and antisera specific to plasma membrane proteins (anti-plasma membrane-bound fatty acid binding protein and sulfo-*N*-succinimidyl oleate) to reduce FA uptake strongly supports protein mediated FA transport (46). Lastly, the observed specificity of FA uptake and the presence of competitive inhibition also suggest the presence of a transport-mediated process (46). The two most notable proteins that have been demonstrated to enhance FA uptake are, fatty acid translocase FAT/CD36 (3, 150) and plasma membrane fatty acid binding protein (FABPpm) (325). While fatty acid transport protein 1 (FATP1) was initially thought to be a FA transporter (290), recent studies have shown that FATP1 is a very long chain fatty acyl-CoA synthetase (79). It is known that both FAT/CD36 and FABPpm are critical for mediating FA transport in skeletal muscle, because blocking of either transporter results in significantly reduced rates of FA uptake (46). While it

is believed that FABPpm and FAT/CD36 may interact with each other to facilitate FA uptake across the sarcolemma, the role of each transporter and whether these FA transport proteins are regulated in concert or independently has not been completely elucidated (44, 47).

The plasmalemmal FABP, or FABPpm, is a 43 kDa peripheral membrane protein and is identical to the mitochondrial isoform of aspartate aminotransferase (325). Because FABPpm is not an integral membrane protein it has been suggested that its primary role may be to mediate the dissociation of FA from albumin although solid evidence supporting this hypothesis is currently lacking (376). The cytosolic, 15 kDa fatty acid binding protein (FABPc) is also an important feature of the FA transport system since it acts as a cytosolic FA sink (46) and may play an important role in the desorption of FA from the plasma membrane into the cytoplasm. In muscle, FABPc is present in great excess and therefore does not limit FA uptake (46). Fatty acid translocase (FAT/CD36) is an integral membrane protein, and is the rat homologue of human CD36. FAT/CD36 is an 88 kDa protein that has two predicted transmembrane domains and is oriented so that it has two short intracellular segments (149). FAT/CD36 is believed to mediate the transport of FA across the plasma membrane although this issue remains contentious as its predicted structure does not fit with that of a typical membrane transporter (376).

Recent studies by Bonen et al. (43, 45, 46, 103) and others (28, 229) have demonstrated that FA transport is a regulatable process, altered by the metabolic demand of skeletal muscle. Reflecting this regulation is the observed expression of FAT/CD36 and FABPpm, which is very high in heart, intermediate in red skeletal muscle and lower in white muscle, thus paralleling the tissues capacity for fat oxidation (44, 46, 212). Increasing the expression of FAT/CD36 in skeletal muscle increases FA transport and oxidation (169), whereas in FAT/CD36 null mice the uptake of FA is reduced (116). Chronic stimulation has been demonstrated to increase the expression of FAT/CD36 and FA transport rates in skeletal muscle (43). While endurance training in rodents failed to demonstrate an increase in the mRNA content of the FA transporters, this may not reflect the level of protein at the plasma membrane as FA uptake with contraction is enhanced (103). In humans endurance training increases FABPpm protein expression (182). Fatty acid transport can also be increased by acute changes in the localization of FAT/CD36 as observed following 30 min of muscle contraction (45) and exposure to insulin (211) which both cause a translocation of FAT/CD36 from an intracellular pool to the plasma membrane. These findings suggest that the acute regulation of FAT/CD36 is similar to the regulation of GLUT-4 in skeletal muscle. Thus, skeletal muscle FA transport can be altered in a number of ways, by chronically altering the expression of FAT/CD36 and FABPpm (chronic stimulation, endurance training, transgenic overexpression) and/or acutely relocating this protein to the plasma membrane (contraction, insulin).

Obesity. In genetic models of rodent obesity and diabetes, transcripts of FAT/CD36, FABPpm and FATP are increased in liver and adipose tissue (28, 29, 229). These models are characterized by either a lack of leptin (*ob/ob* mice), or leptin receptor defects (*db/db* mice and obese Zucker *fa/fa* rat). Therefore, it seems plausible that the lack of leptin interaction with skeletal muscle leads to the overexpression of FAT/CD36 and FABPpm, and the accumulation of intramuscular TG observed in obesity. In obese Zucker rats (*fa/fa*) that are resistant to leptin, skeletal muscle FA transport is markedly increased, due to a relocation of FAT/CD36 to the plasma membrane, in the absence of any changes in its total expression (210). Plasma membrane FABPpm is also increased in red skeletal muscle of obese Zucker rats (338). In obese humans, which characteristically have elevated circulating leptin levels, but are hypothesized to be leptin resistant, skeletal muscle FABPpm mRNA is increased (305) although care must be taken when extrapolating this data to levels of protein expression. Therefore, a common defect in skeletal muscle of both obese rodents and humans appears to be the overexpression of the FA transport proteins at the level of the plasma membrane. This increased potential for FA uptake in the presence of unaltered or blunted FA oxidation (as discussed below) may lead to the accumulation of intramuscular TG common in obesity.

iii) Fatty Acid Oxidation

During the fasting (postabsorptive) condition, lipid oxidation is the predominant metabolic activity of resting skeletal muscle (93). A reduction in skeletal muscle FA oxidation has been postulated to promote positive fat balance and lead to the accumulation of intramuscular TG common in obesity (178, 226). Therefore, in this section the potential sites of FA regulation/dysregulation in obesity will be discussed.

a) Uncoupling Proteins and Mitochondrial Proton Leak

The chemiosmotic theory, proposes that as electrons are passed down, the energy gradient of the electron transport chain, protons are pumped out of the inner matrix of the mitochondria, generating an electrochemical gradient (proton motive force) across the inner mitochondrial membrane (128). These protons have two probable fates: They can reenter the mitochondrial matrix through ATP synthase, driving the synthesis of ATP and the consumption of oxygen; this is referred to as coupled respiration. Alternatively, protons may “leak” back across the membrane in a manner not linked to ATP production resulting in fuel oxidation with the production of heat (4). This is referred to as uncoupled respiration (128). Proton leak from rodent skeletal muscle mitochondria has been estimated at 20-50% of resting metabolic rate (132, 151). Therefore, variations in skeletal muscle proton leak between individuals may play an important role in the development of obesity (51, 95, 101, 160, 209, 277).

While cold-induced thermogenesis in rodents has long been known to involve brown adipose tissue and a mitochondrial uncoupling protein (now known as UCP-1) (277) the discovery in both rodents and humans of new members of the UCP family, ubiquitously expressed UCP-2 (120) and skeletal muscle specific UCP-3 (54, 135), has prompted a re-examination of the molecular mechanisms of thermogenesis and their potential contribution to the pathogenesis of obesity. It is well documented that UCP-1 increases proton leak in brown adipose tissue, and it was originally proposed that due to their high sequence homology UCP-2 and -3 (59 and 57% respectively), they would also have the same function. In support of this hypothesis, several studies in yeast and mammalian cell lines have demonstrated increased proton flux and decreased growth rates when UCP-3 is overexpressed (119, 127). Indirect evidence supporting the role UCP-3 in skeletal muscle comes from UCP-3 knockout mice which have an increased state 3/state 4 ratio, indicating increased coupling (therefore decreased leak) (77). Despite these findings the importance of UCP-2 and UCP-3 expression in the regulation of energy expenditure remains equivocal due to several studies, which do not support a physiological role of UCP-2 or UCP-3 in mediating mitochondrial uncoupling: 1) the tissue distribution of the UCP homologues (both protein and mRNA) does not match the proton leaks measured, i.e. some tissues high in UCP-2 have a low proton leak and vice versa (132), 2) UCP-2 mRNA is increased several fold in brown adipose tissue of UCP-1 knockout mice but no change in uncoupling

activity is observed (132), 3) UCP-3 knockout mice are not obese and exhibit normal responses to fasting and thyroid hormone (137) and 4) while fasting induces a 4-5 fold increase in both UCP-2 and UCP-3 mRNA and a 2 fold increase in UCP-3 protein levels, leak-dependent H^+ conductance in isolated skeletal muscle mitochondria remains unchanged (31).

The paradox between the upregulation of UCP homologues with starvation (53, 131, 135, 206) has led to the alternative hypothesis that the primary role of UCP-2 and -3 is to up regulate FA metabolism in skeletal muscle (51, 101, 151). During starvation, the low insulin and high epinephrine results in a net increase in FA release which is associated with muscle UCP-3 up regulation and enhanced FA oxidation, whereas during refeeding there is a reduction in circulating FA resulting in the down regulation of UCP-3 in skeletal muscle and reduced FA oxidation (101). Furthermore, numerous studies have demonstrated increased UCP-3 expression following high-fat feeding (53, 136) and the upregulation of UCP-3 by FA (168, 342) independent of fasting. Lastly an acute bout of endurance exercise increases UCP-3 mRNA and protein expression (52, 131, 161, 258, 334, 335, 371) and this has recently been demonstrated to be solely in response to elevated FA (293). These findings suggest that while UCP-3 may cause mitochondrial proton leak under some situations it may have more important functions as a mitochondrial transporter of FA anions (101, 304). In an alternative model Himms-Hagen and Harper (160) suggest that UCP homologues are coupled with

mitochondrial acyl-CoA thioesterase, which in turn results in the production of free CoA (CoASH). While these hypotheses are entirely speculative, they do provide alternatives to proton leak suggesting that the primary role of the UCP homologues may be as mediators of nutrient flux and regulators of FA metabolism.

Obesity. The relationship between UCP-2 and -3 mRNA and protein expression and obesity and type 2 diabetes remains unknown despite several studies investigating this relationship (291). For instance in skeletal muscle of the obese UCP-2 mRNA expression has been reported to be higher (304), equal (296), or lower (346). In addition while one study was able to establish a reciprocal relationship between UCP-2 mRNA content in skeletal muscle and BMI, body fat content and respiratory quotient other studies have failed to identify such a relationship (291). In addition UCP-3 mRNA expression does not appear to be altered by obesity (131) but protein expression is negatively correlated to the development of type 2 diabetes (292). Although a number of different amino acid substitutions have been reported in the *UCP-3* gene, most are rare and therefore polymorphisms in *UCP-3* do not make a large contribution to the susceptibility of obesity (95). Therefore, despite the significant interest in this area of research the contribution of UCP-2 and -3 in the regulation of skeletal muscle FA metabolism and their role in the development of obesity remains largely unknown. One of the reasons for the inconclusive findings may be because almost all studies have

investigated expression of mRNA rather than protein and therefore do not take into account possible posttranscriptional regulation. The recent production of specific and sensitive antibodies will hopefully provide a clearer understanding of the role of UCP-2 and -3 in human obesity and skeletal muscle FA metabolism.

b) The CPT System: AMPK, ACC2 and Malonyl-CoA

Due to their important role in the regulation of nutrient flux in skeletal muscle, AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC2) and malonyl-CoA have been described as metabolic fuel sensors that regulate the partitioning of fatty acyl-CoA towards oxidation or storage and have been suggested to play an important role in regulating FA metabolism in obesity (281, 365). Carnitine palmitoyl transferase-1 (CPT 1) catalyzes the rate-limiting step for the transport of FA across the mitochondrial membrane for β -oxidation, and is considered the most important regulatory enzyme in determining the cellular rate of FA oxidation (312). Malonyl-CoA, generated by ACC2, is a key metabolite in the regulation of energy homeostasis as it potently inhibits CPT-1 and therefore FA oxidation. Both glucose and insulin increase the activity of ACC2 in rodent and human skeletal muscle by increasing the cytosolic concentration of citrate, an allosteric activator of ACC2 and a precursor of its substrate, cytosolic acetyl-CoA, leading to increased malonyl-CoA concentrations (23, 100). High-fat feeding in humans reduces resting malonyl-CoA (249). The turnover of malonyl-CoA is controlled by the activity of malonyl-CoA decarboxylase and although it was

originally shown to be activated by AMPK (285) a recent report demonstrates that this is not the case (142). Exercise increases AMPK activity thereby inhibiting ACC2 activity and reducing malonyl-CoA concentrations in rodent skeletal muscle (68, 96, 167, 345, 363, 364). Exercise also activates AMPK and phosphorylates ACC2 in human skeletal muscle (68, 318) as well as muscle from type 2 diabetics (243) but during exercise in humans malonyl-CoA concentrations do not change (96, 248-250, 312). Part of this discrepancy between malonyl-CoA in rodents and humans may be due to the nature of the measurement of malonyl-CoA, which is made in total tissue homogenates. Because ACC2 by virtue of its unique NH₂-terminal extension appears to associate with the outer mitochondrial membrane, possibly in close proximity to CPT-1 (1), changes in malonyl-CoA concentrations might be of much greater magnitude than those measured in total tissue homogenates and since malonyl-CoA concentrations are much lower in human skeletal muscle compared to rat skeletal muscle changes may be difficult to detect. Therefore due to their central role in regulating FA oxidation the measurement of ACC2 and AMPK may more accurately reflect changes in FA oxidation than the measurement of malonyl-CoA. Indeed several authors have suggested that defects in the AMPK signaling pathway may play an important role in the development of obesity, but evidence of defective AMPK signaling in the obese does not exist (281, 365).

Skeletal muscle content of malonyl-CoA is increased in animal models of obesity

(280). In mice lacking ACC2, which are lean and hyperphagic, malonyl-CoA is reduced 30-fold in skeletal muscle which is associated with an increase in soleus muscle FA oxidation by 30% under basal conditions and 45% in the presence of insulin thus demonstrating the critical role of malonyl-CoA in the regulation of FA metabolism in rodents (2). Furthermore, FA stores in adipose tissue and liver were markedly decreased thus demonstrating the significant effect that reduced malonyl-CoA levels can have on whole body adiposity (2). Several authors (176, 185, 305) have reported reduced CPT-1 activity in the obese but this reduction in CPT-1 activity is proportional to an overall reduction in activity of the oxidative enzymes citrate synthase, cytochrome C oxidase and β -hydroxyacyl dehydrogenase; marker enzymes of the Krebs cycle, electron transport, and β -oxidation, respectively. Therefore reductions in CPT-1 activity likely reflect reduced mitochondrial content rather than a specific impairment for FA oxidation. While the potential role of malonyl-CoA in the regulation of FA metabolism in human skeletal muscle is still unclear (248-250, 312) it is apparent that sensitivity of CPT-1 to malonyl-CoA can be altered as it is increased with endurance training (314). Thus it may be possible that CPT-1 sensitivity to malonyl-CoA is increased in obesity although this is untested.

iv) Fatty Acid Esterification

The biosynthetic pathways responsible for intramuscular TG synthesis (See Figure 1) are generally poorly understood in skeletal muscle (343). The synthesis of TG is regulated in part by the supply of the substrates glycerol-3-phosphate (from carbohydrate metabolism) and fatty acyl-coenzyme A supplied through intracellular hydrolysis and/or transport from exogenous sources (343). Due to the low activity of glycerol kinase in skeletal muscle direct phosphorylation of glycerol contributes little to the formation of glycerol-3-phosphate (343).

Therefore, the first committed and rate-limiting step in the biosynthesis of TG is the acylation of *sn*-glycerol-3-phosphate to form 1-acyl-*sn*-glycerol-3-phosphate by glycerol-3 phosphate acyltransferase (GPAT) (83, 89). As might be expected from an enzyme responsible for synthesising TG, GPAT activity is increased in skeletal muscle of obese rodents. It is believed the control of GPAT likely involves insulin because in hypoinsulinemic states (Type I diabetes, starvation) GPAT activity is decreased while insulin injection increases GPAT mRNA by 2-fold within an hour and 19-fold within 6 hours (83, 89). AMPK activation has also been shown to phosphorylate GPAT thereby reducing its activity (240).

Taken together these findings suggest that increased activity of GPAT may contribute to the accumulation of intramuscular TG in the obese.

Figure 1. Overview of TG metabolism in skeletal muscle. 1. glycerol-3-phosphate dehydrogenase; 2. glycerol-3-phosphate acyltransferase; 3. 1-acylglycerol-3-phosphate acyltransferase; 4. phosphatidic acid phosphatase; 5. diacylglycerol acyltransferase; 6. hormone sensitive lipase; 7. diacylglycerol lipase; 8. mono-acylglycerol lipase; 9. glycerol kinase (not present in skeletal muscle); Pi , inorganic phosphate (from van der Vusse and Reneman (1996)).

v) TG hydrolysis

The breakdown of intramuscular TG is accomplished through sequential hydrolysis of the three FA residues and is catalyzed by three distinct lipases (See Figure 1). Muscle and heart cells have low glycerokinase activities; therefore free glycerol formed through lipid hydrolysis is not recycled for synthesis but is rather released or oxidized (343). The hydrolysis of TG to DG is the rate-limiting step in TG breakdown, since the other two lipases have much higher activities (343). For more than a decade it was believed that the breakdown of TG was regulated by lipoprotein lipase (LPL), but the role of LPL in TG breakdown in skeletal muscle was questioned because 1) in muscle cells the enzyme is produced as a secretory protein and resides in vesicles and 2) the optimal pH for LPL is 8.5 while resting human muscle has a pH of ~7. These findings suggested that muscle may contain the same neutral lipase which controls lipolysis in adipose tissue and is known as hormone sensitive lipase (HSL). With the aid of antibodies and cDNA probes the expression of HSL in skeletal muscle was established (162). A subsequent study which isolated individual skeletal muscle fibers confirmed that HSL was indeed present within myocytes and not the adipocytes interlaced between fibers (309). It was later confirmed that HSL is the key-rate limiting enzyme in the breakdown of intramuscular TG in skeletal muscle (163) although a recent study in HSL deficient mice also highlights its role in the catabolism of DG (143). Both contraction (195) and epinephrine (196) increase the activity of HSL in skeletal muscle. While it is known that adipose HSL activity is increased by

phosphorylation (12, 254) the mechanisms regulating skeletal muscle HSL remains unknown. Nothing is known about the regulation of skeletal muscle HSL in obesity. In HSL deficient mice, there is an accumulation of DG within the skeletal muscle (143) which has also been observed in obese humans (the connection between DG and insulin resistance is discussed in detail below) therefore, it seems possible that decreased HSL activity in the obese may lead to the accumulation of DG but direct evidence of this does not exist. In adipose tissue, both *in vivo* and *in vitro* studies have shown that the lipolytic effect of catecholamines, is blunted in obese subjects (197).. The same may also be true for skeletal muscle HSL, but clearly more research is needed to understand the biochemical regulation of HSL in skeletal muscle and to determine if there are differences in HSL phosphorylation and therefore levels of activity between lean and obese individuals.

C) Obesity and Skeletal Muscle Insulin Resistance

i) Introduction

It is estimated that by the year 2020 there will be approximately 250 million people affected by type 2 diabetes worldwide (302). The primary factor responsible for the development of type 2 diabetes is skeletal muscle insulin resistance (302). Although much is known about the pathways of glucose metabolism and the molecular factors that mediate the action of insulin, the strong association between obesity and insulin resistance remains largely unknown.

ii) Mechanisms

One of the most intensely studied theories on the development of insulin resistance with the onset of obesity is the hypothesis proposed by Randle et al (273) close to forty-years ago, that high levels of FA (common in obesity) would inhibit glucose metabolism and lead to the development of type 2 diabetes. The main features of this model, demonstrated in rat heart and diaphragm, was that increased fat oxidation (in the presence of elevated FA) lead to the accumulation of acetyl-CoA and citrate, which in turn would inhibit both pyruvate dehydrogenase (PDH) and phosphofructokinase respectively. This inhibition of the glycolytic pathway would lead to increased glucose-6-phosphate concentrations, which in turn would inhibit hexokinase. Randle's initial hypothesis has led to a multitude of studies in both rodents and humans and while elevated FA have been demonstrated to inhibit insulin-stimulated glucose uptake in skeletal muscle as predicted, the mechanisms

of action were not as originally described by the glucose-fatty acid cycle (178, 302, 311).

Acutely, in rodents (141) and humans (99), an artificial elevation of FA has been demonstrated to reduce glucose transport by abolishing insulin-stimulated IRS-1 associated PI 3-kinase activity. It's important to note that reduced glucose transport following elevated FA was associated with reduced intracellular glucose concentrations rather than elevated glucose and glucose-6-phosphate concentrations as would be predicted by the glucose-fatty acid cycle, thus also highlighting the direct effects of FA on glucose transport (302). This reduced glucose transport activity may be due to a direct effect of intracellular FA (or some FA metabolite) on PI 3-kinase, or may be secondary to alterations in upstream insulin signaling events. Recent evidence in rodents provides support for the latter as a reduction in insulin-stimulated IRS-1 tyrosine phosphorylation was associated with activation of protein kinase C, a known serine kinase that inhibits IRS-1 phosphorylation and is activated by diacylglycerol (DG) (88, 141, 171).

The chronic dysregulation of FA metabolism in skeletal muscle of the obese (as discussed previously) is also believed to play an important role in the development of type 2 diabetes. In the past several years a large number of studies have demonstrated that increased content of skeletal muscle TG in muscle is directly related to reduced insulin sensitivity in skeletal muscle of the obese (138, 139,

156, 176-178, 214, 215, 256). The mechanisms by which elevated intramuscular TG reduce insulin sensitivity has been shown to involve blunted tyrosine phosphorylation of the insulin receptor and insulin receptor substrate PI-3-kinase (347). Caution must be exercised when interpreting the relationship between elevated TG and reduced insulin sensitivity though, as endurance training has been reported to increase muscle TG (164, 238) while also improving insulin sensitivity (222). This suggests that muscle TG may not have adverse metabolic consequences in muscle that has a capacity for efficient lipid utilization. More recently, ideas have emerged in the literature suggesting that increases in TG are likely not the principle cause of insulin resistance, as this neutral fat is probably relatively innocuous, but instead elevated TG may act as a surrogate marker for some other FA derived entities, such as fatty acyl-CoA or DG (67, 177, 178, 226). Several authors have since reported a strong association between fatty acyl-CoA and reduced insulin sensitivity in both humans and rodents (247, 333). Elevated concentrations of fatty acyl-CoA in muscle may also increase the concentration of DG, which results in the translocation, and activation of protein kinase C (PKC), which as discussed previously, may inhibit insulin receptor phosphorylation and therefore reduce insulin sensitivity (25, 141). Lastly, other authors have suggested that changes in the FA composition of the plasma membrane may alter insulin sensitivity by inhibiting proper GLUT-4 transporter docking (50). While the exact mechanism by which lipids reduce insulin sensitivity remains unclear, it is clear

that perturbations of skeletal muscle FA metabolism in obesity are a major factor in the development of insulin resistance.

iii) High-fat Diets and Insulin Sensitivity in Rodents

A common model used to study the development of insulin resistance is the feeding of high-fat diets (30, 32, 48, 126, 145, 146, 157, 170, 186, 194, 205, 278, 321, 359, 361). High-fat diets have been shown to increase energy intake due to their minimal appetite-suppressing effect and because of their high palatability (294, 320). In addition, high-fat diets have a very low thermic effect therefore a positive energy balance often results following high-fat feeding (359). In as little as two days of high-fat feeding there are changes in whole-body and skeletal muscle insulin sensitivity in rodents (184). There is also evidence of hyperinsulinemia, hyperglycemia, and if the diet is continued for extended periods of time, diabetes in genetically susceptible strains of rodents (67, 146, 170, 361). It is important to note that these effects are seen in the absence of obesity, that is when animals are calorically matched/paired with low fat fed controls indicating a direct effect of the high-fat diet (67, 146, 170, 186, 361). While, it was initially hypothesized that high-fat diets may change membrane fluidity resulting in insulin receptor downregulation or the inhibition of glucose transporter function (157) it has been demonstrated that this not the case but instead high-fat feeding has been found to reduce insulin-stimulated tyrosine phosphorylation, which in turn results in reduced GLUT-4 translocation to the plasma membrane (145, 146, 375). High-

fat feeding and insulin resistance is associated with an increase in intramuscular TG (324) but as discussed above this may simply be a marker for the presence of more deleterious FA derivatives. Consistent with this notion, Bell et al. (25) found that the rapid reversal of skeletal muscle insulin resistance in high fat-fed rats after a single high-carbohydrate meal was associated with a fall in muscle fatty acyl-CoA levels and loss of membrane bound PKC θ activity. Therefore, in summary high-fat diets appear to reduce insulin sensitivity by increasing fatty-acyl-CoA and DG accumulation in skeletal muscle resulting in the translocation of PKC θ leading to a downregulation of insulin-stimulated tyrosine phosphorylation and therefore GLUT-4 translocation and glucose transport.

The results of high-fat diet experiments depend largely on the fat composition of the diet because when substitution of a portion of the fat from a high polyunsaturated (n-6) fat diet (45-60% total kcal as fat) is replaced with fish oil containing long-chain n-3 FAs, glucose uptake in skeletal muscle and hepatic glucose output are restored to levels observed in the low-fat fed control rats (15, 90, 173, 200, 320, 322-324). Ad libitum feeding of rodents with high-fat diets containing fish oil reduces body mass gain, body fat mass, and limits abdominal and epididymal adipose tissue hypertrophy (257). Therefore, improved insulin sensitivity in skeletal muscle may be related to reduced obesity, more specifically visceral obesity, which is strongly associated with the development of insulin resistance in rodents and humans (21, 22, 80, 188, 236, 279). Also reduced

adipose hypertrophy with fish oil feeding may also prevent the release of several newly discovered, adipocyte derived hormones such as resistin (121, 301, 319), adiponectin (148, 329) and tissue necrosis factor- α (262) all of which have been demonstrated to cause insulin resistance in rodent skeletal muscle. More research is needed investigating the effects of n-3 FA on the release of these hormones.

Alternatively, improved insulin sensitivity following fish-oil feeding has also been attributed to reduced intramuscular TG in skeletal muscle (247, 322) as well as reduced levels of fatty acyl-CoA and a trend towards lower DG content (247). This reduction in TG/fatty acyl-CoA levels in skeletal muscle has been attributed to the potent effects of n-3 FA on stimulating peroxisome proliferator activating receptor- α (PPAR α) and enzymes of FA oxidation in hepatic tissue (247). Polyunsaturated FA have also been demonstrated to stimulate UCP-2 expression via PPAR α in hepatic tissue (13) and although the effects of fish oil on UCP-2 and -3 expression in skeletal muscle has not been investigated, it seems a likely possibility that they may be upregulated. In a separate study a high-fat diet enriched in n-3 FA maintained insulin receptor concentrations, insulin receptor tyrosine phosphorylation and PI-3 kinase activity and total GLUT-4 content in skeletal muscle (330). In summary these two recent studies suggest that stimulation of PPAR α by fish oils reduces the accumulation of TG/fatty acyl CoA in skeletal muscle, which in turn allows for the maintenance of normal insulin signaling. These results suggest that the type of fat in the diet may be more

critical then the overall percentage of fat and once again highlight the important role the accumulation of lipids (TG, fatty acyl-CoA and/or DG) may have in the development of skeletal muscle insulin resistance.

iv) High-fat Diets and Insulin Sensitivity in Humans

Despite the overwhelming evidence supporting the role of fish oil in preventing the development of insulin resistance in rodents, studies in humans have not shown as dramatic results. In humans, substitution for 3 weeks with 6 g/day of fish oil leads to a reduction in body fat mass and leads to an increase in basal lipid oxidation (90) while reducing glucose and insulin concentrations following a carbohydrate load (97). Although positive results have been shown in two investigations using fish oil supplements (115, 268), two studies have failed to show improvement in insulin action (49, 133), indicating that the results of short-term fish oil supplementation in humans are equivocal. One possible explanation for the contrasting results between rodents and humans may be due to the fact that, while fish oil prevents the development of insulin resistance in nondiabetic rats, in humans the efficacy of fish oil supplementation has been measured by its ability to reverse insulin resistance and type 2 diabetes (320).

In contrast to the effects of short term high-fat diets in humans (125), there is now accumulating evidence from cross-sectional and epidemiological studies which appear to link high fat, and particularly saturated fat, intake with insulin resistance

and the development of type 2 diabetes (320). In parallel with these observations the long-term high fat intake of fish oils has been associated with the relative reduction in the incidence of glucose intolerance (117). This improved insulin sensitivity has been attributed to a clear and consistent relationship between the percentage of highly unsaturated lipids in the muscle structural membrane and insulin action (50). Conversely, the more saturated the muscle membrane phospholipids, the more insulin resistant the individual (50). Despite the beneficial effect of dietary polyunsaturated fats on insulin action, more recent studies have also established a relationship between the dietary n-6 to n-3 ratio, that is the higher the n-6/n-3 ratio, the higher the fasting insulin (320). In addition, reducing the dietary n-6/n-3 ratio has been shown to have a beneficial effect on glycaemic control in type 2 diabetic subjects (112). Therefore, it appears that long term supplementation of the diet with n-3 FAs may have beneficial effects on insulin sensitivity in humans.

D) Leptin and the Importance of Skeletal Muscle

i) Acute Effects of Leptin on Carbohydrate Metabolism

Skeletal muscle is quantitatively the most important target tissue for insulin in glucose metabolism and therefore plays a central role in insulin action and in the pathogenesis of insulin resistance. Early studies in *ob/ob* mice that were obese and diabetic demonstrated that recombinant leptin injection decreased hyperglycaemia and hyperinsulinaemia independent of alterations in food intake or before there was a measurable loss of body mass (260). Despite the large number of studies conducted following this initial report the effect of acute leptin treatment on skeletal muscle glucose uptake measured both *in vivo* and *in vitro* remains ambiguous with reports suggesting either increased (64, 174, 175, 251) or no change (59, 232, 274, 374) in glucose uptake. Equivocal results following acute leptin treatment have also been found in regard to glycogen synthesis and glucose turnover (175, 199, 241, 242). It has been suggested that an intact sympathetic nervous system may be a prerequisite for leptin to exert an acute effect on glucose uptake. This is supported by a general failure of leptin to elicit a stimulatory effect on glucose uptake in denervated muscle (174) or when norepinephrine release is blocked (147).

Adding to the complexity of this issue is the recent report that leptin acutely increases AMPK both *in vivo* and *in vitro* in rodent skeletal muscle (234).

Although glucose uptake was not measured in this study other studies in isolated

(14, 155, 244, 252) or perfused skeletal muscle (230) and also in conscious rats (26) have demonstrated increased glucose uptake and the translocation of GLUT-4 to the plasma membrane following activation of AMPK by AICAR. Activation of AMPK by AICAR, contraction or hypoxia has also been demonstrated to acutely increase UCP-3 protein expression in rodent skeletal muscle (371). Increases in UCP-3 expression have been demonstrated to stimulate glucose uptake due to an increase in GLUT-4 translocation in muscle cells (166). This effect is mediated through a PI-3 kinase-dependent mechanism (166). These findings when summarized suggest that leptin activation of AMPK in skeletal muscle should lead to an increase in glucose uptake. A possible explanation for the equivocal results surrounding the effects of leptin on glucose uptake may have to do with the kinetics of the leptin response (187, 328). Supporting this idea is the recent finding (234) that intravenous leptin injection in rodents produces a biphasic response in AMPK activity resulting in a 2-fold increase at 15 min, a return to baseline by 60 min, and a second 2-fold elevation at 6 hours. Also, the effects of leptin on AMPK activity were also greater in red (slow-twitch, oxidative) vs. white (fast-twitch, glycolytic) skeletal muscle (234). This suggests that studies that have failed to detect alterations in glucose uptake and glycogen synthesis may have measured glucose uptake approximately 60 min after treatment and secondly, may have measured glucose uptake in glycolytic muscle fibers. Clearly, this is an area with profound implications for the treatment of diabetes and therefore it can be expected that in coming months considerable insight into the role of leptin and

glucose uptake will be made.

ii) Acute Effects of Leptin on Fatty Acid Metabolism.

Injection of recombinant leptin in *ob/ob* mice results in the rapid, selective, depletion of adipose tissue while maintaining lean body mass. As stated previously the primary tissue by virtue of its mass capable of metabolising FA is skeletal muscle. In 1997, Muoio et al. (241) were the first to demonstrate that leptin acutely increases FA oxidation while reducing FA esterification in resting rodent skeletal muscle. Previously, studies conducted in pancreatic islets demonstrated the potent effects of leptin gene transfer in reducing TG storage. This reduction in TG storage was associated with an increase in the mRNA of enzymes of FA oxidation such as UCP-2 and CPT-1 while reducing the expression of ACC2 and GPAT (373). As discussed previously, this stimulatory effect on FA oxidation has recently been demonstrated to be mediated through leptin activation of AMPK, which in turn reduces ACC2 activity (234). This increase in AMPK activity would be expected to increase UCP-3 protein expression (371). Although levels of UCP-3 protein have not been measured in skeletal muscle following acute leptin treatment, several studies have demonstrated increased UCP-3 mRNA following acute leptin treatment (131, 135, 206). While the role of UCP-3 as an uncoupler of oxidative phosphorylation has been questioned, significant evidence implicates a substantial role of UCP-3 in altering fuel selection to favor FA oxidation in skeletal muscle (101, 160, 304). In addition

AMPK has been demonstrated to inhibit the activity of GPAT thereby limiting TG esterification (240). Therefore, it is currently believed that leptin repartitions FA towards oxidation and away from storage in skeletal muscle through activation of AMPK which may increase UCP-3 protein expression and by reducing the activity of ACC2, while also reducing the activity of GPAT leading to decreased TG esterification.

iii) Chronic Effects of Leptin on Skeletal Muscle Carbohydrate and Fatty Acid Metabolism.

While the results of acute leptin treatment on glucose transport are equivocal, chronic hyperleptinemia (>48 hours) clearly increases glucose uptake in skeletal muscle (58, 70, 308, 350, 366, 367). However, this is not due to increased GLUT-4 expression (367). A possible mechanism for improved insulin sensitivity in rodents may be the decrease of intramuscular TG (or other factors related to decreased TG, i.e. fatty acyl-CoA/DG) observed in oxidative skeletal muscle following leptin treatment, since intramuscular TG accumulation is correlated with insulin resistance as discussed previously. While these effects are well documented the mechanisms regulating this reduction are unknown but may be broadly postulated to involve one of a combination of reduced FA transport, increased FA oxidation, reduced FA esterification or increased TG hydrolysis. Similar observations in regard to the insulin sensitizing effects have also been observed in humans with low serum leptin levels (<4 ng/ml). In these patients,

elevating circulating leptin to normal levels (11.1 ng/ml) via recombinant leptin injection for 4 months, improved glycaemic control (as assessed by an insulin-tolerance test and an oral glucose tolerance test) and led to the discontinuation or a large reduction in diabetic drug therapy (253). In addition, recombinant leptin treatment reduced liver TG by 40% as measured by magnetic resonance imaging (253). Unfortunately, skeletal muscle TG content was not measured in these subjects. These findings suggest that chronically elevated leptin levels may reduce intramuscular TG content and associated fatty acid metabolites, which in turn may result in improved insulin sensitivity.

E) Obesity and Leptin Resistance

Although leptin administration in *ob/ob* mice has been demonstrated to cause a rapid reversal of obesity through a reduction in caloric intake and an increase in basal metabolic rate, the relevance of this genetic model to the treatment of human obesity appears to be minimal. In contrast to the leptin deficient *ob/ob* mice high levels of circulating leptin even when normalized per kilogram fat mass characterize human obesity. Therefore, despite the presence of elevated leptin concentrations, which should reduce food intake and body fat, obese persons appear to be insensitive or resistant to leptin and continue to maintain high levels of body fat. In addition, while recombinant leptin injection reduces body fat in obese rodents the effects appear to be minimal in obese humans. In the first clinical trial examining the efficacy of leptin treatment Heymsfield et al. (159) found that while recombinant leptin injection at high doses (elevated by ~20 fold) resulted in weight loss due primarily to lower energy intake (as assessed by 48-hour dietary recall, a relatively insensitive measure) there was considerable variability in the amount of body mass lost between subjects at any given dose. A more controlled follow up study which used doubly labeled water and a respiration chamber to measure metabolic rate while also assessing food intake and appetite profiles using the Universal Eating Monitor and the Three Factor Eating Questionnaire respectively confirmed that while recombinant leptin injection reduces appetite it has no effect on energy expenditure or basal metabolic rate (360). This data suggests that the primary site of leptin resistance may be in

metabolically important tissues such as skeletal muscle (360).

Several postreceptor inhibitors of leptin action have been identified. One of these, SH2-containing phosphatase 2 (SHP-2) inhibits STAT-3 mediated gene induction by dephosphorylating activated OB-R (33). A second inhibitor named "protein inhibitor of activated STAT" (PIAS3), blocks the DNA-binding activity of STAT-3 (74, 259). Lastly, a mediator of leptin resistance that has demonstrated great potential is the recently identified member of the suppressors of cytokine signaling family, SOCS-3. SOCS-3 was first identified in the hypothalamic nuclei of leptin resistant A^y/a mice (35) but is also expressed in liver, small intestine, white adipose tissue and skeletal muscle. SOCS-3 protein expression potently inhibits leptin signaling by suppressing STAT-3 activation at TYR-987 and TYR-1077 motifs within the cytoplasmic domain of the leptin receptor (18, 34, 111, 113). In further support of its role as an inhibitor of leptin action, SOCS-3 expression is induced in the arcuate and dorsomedial hypothalamic nuclei as well as the liver and small intestine (111) of mice after leptin treatment (34, 111). A recent report also indicates that both insulin and tumor necrosis factor- α (TNF- α), two polypeptides known to be elevated with obesity, induce SOCS-3 mRNA expression in liver, white adipose tissue and skeletal muscle of mice (110). In support of the role of TNF- α , mice lacking TNF- α receptors have a pronounced decrease in SOCS-3 expression in white adipose tissue compared to *ob/ob* mice. The role of SOCS-3 in human obesity has yet to be demonstrated.

Several studies have shown that chronic high-fat feeding in rodents leads to an increase in plasma leptin levels. Despite large increases in plasma leptin levels obesity still develops in susceptible strains of rodents, thus suggesting the development of leptin resistance following high-fat feeding. In rats made obese by a high-fat diet, intraperitoneal injection of recombinant leptin has no effect on food intake and body mass, but leptin injected directly into the central nervous system results in appetite suppression and loss of body fat. This finding suggests that leptin resistance may be due to 1) reduced transport of leptin across the blood brain barrier (BBB) following high-fat feeding and/or 2) peripheral leptin resistance in metabolically important tissues such as skeletal muscle.

Evidence supporting reduced leptin transport across the blood brain barrier following high-fat feeding as a potential site of leptin resistance has been demonstrated by Burguera et al. (60). In this study high-fat feeding (40% kcal) for 10 weeks reduced leptin transport across the BBB by 3 fold, a reduction similar to that seen in genetically obese *fa/fa* Zucker rats. Measurement of ObR-S, the predominant isoform expressed within the BBB and hypothesized to transport leptin, has been found to be unaltered (108) or increased (38) following high-fat feeding. Thus, the mechanism underlying reduced leptin transport across the BBB does not involve reduced expression of the putative leptin transporter within the BBB. Instead this suggests that the ObR-S may be more easily saturated or has

reduced activity following high-fat feeding, but direct evidence of this hypothesis does not exist. Alternatively, evidence of hypothalamic leptin resistance independent of leptin transport across the BBB has also been demonstrated following 15 weeks of high-fat feeding, which reduced STAT-3 activation by 75% following intracerebroventricular injection of leptin (108). These results suggest that central leptin resistance induced by a high-fat diet has at least two independent causes: 1) an apparent defect in the transport of leptin across the BBB and 2) an intracellular signaling defect which lies upstream of STAT-3 and reduces its activation by leptin.

Despite the known effects of leptin on peripheral tissues very few studies have investigated the effects of leptin resistance in peripheral tissues and in particular skeletal muscle. Recently, one study has demonstrated the development of hepatic leptin resistance following just 3 days of high-fat feeding as evidenced by the inability of leptin to stimulate hepatic gluconeogenesis (349). In white adipose tissue 8 weeks of high-fat feeding (60% kcal) led to an increase in SOCS-3 mRNA and protein expression and although a direct measure of leptin resistance was not determined, overexpression of SOCS-3 (by an amount approximately equal to that seen in WAT) in pancreatic islets reduced the ability of leptin to stimulate FA oxidation by 75% (352). This suggests that increased SOCS-3 expression following high-fat feeding could block leptin's ability to stimulate FA oxidation. Surprisingly, another study failed to show an increase in SOCS-3 or PIAS-3

mRNA in the hypothalamus, white or brown adipose tissue, heart, liver and skeletal muscle of rats fed a high-fat (42% kcal) diet for 15 weeks (259). The reason for this inconsistency is not known, but may be due to the fact that only mRNA measurements were made suggesting post-transcriptional regulation of SOCS-3 and PIAS-3. In addition, SOCS-3 expression in white adipose tissue is also elevated in old rats compared to young rats (353), which may explain the apparent development of leptin resistance with aging in some strains of rats (203).

These findings in rodents suggest that the development of skeletal muscle leptin resistance (perhaps mediated by SOCS-3) may be an important contributor to the development of obesity. In addition, skeletal muscle leptin resistance could also lead to the development of type 2-diabetes by inhibiting leptin's effects on FA partitioning leading to an increased content of intramuscular TG and therefore insulin resistance. Therefore, it is possible that during the development of human obesity, skeletal muscle becomes resistant to leptin, leading to the accumulation of intramuscular TG and other deleterious metabolites, and the development of insulin resistance.

Chapter 2: Statement of the Problem and Rationale for Studies

The primary purpose of this thesis was to examine the effects of leptin (chronic and acute) on skeletal muscle FA metabolism.

In the first portion of this thesis, we investigated the chronic effects of leptin. Previously, chronic leptin treatment (>2 days) has been demonstrated to reduce skeletal muscle intramuscular TG, but the mechanisms regulating this reduction are unknown. However, they may be broadly postulated to be due to several factors, including 1) reduced FA uptake 2) a repartitioning of incorporated FA towards oxidation and away from esterification, and 3) an increased rate of TG hydrolysis. Therefore, I examined the effects of 14 days of physiological hyperleptinemia on a) FA metabolism and b) FA transport. FA metabolism was measured in isolated soleus muscle using the pulse-chase technique, which allows for the simultaneous determination of endogenous and exogenous rates of FA oxidation as well as esterification and hydrolysis of intramuscular lipids. To determine the mechanisms of leptin action on FA oxidation and TG hydrolysis, we measured the activity of the oxidative markers citrate synthase (CS) and β -hydroxyacyl-CoA dehydrogenase (β -HAD) and the protein expression of HSL, respectively. To determine rates of FA uptake following chronic leptin treatment giant sarcolemmal vesicles were prepared from red and white skeletal muscle and the mRNA and protein expression of FAT/CD36 and FABPpm were subsequently measured.

Despite the pronounced effects of both acute and chronic leptin treatment on peripheral tissues (i.e. skeletal muscle) in rodents, the relevance of leptin treatment in obese humans has been questioned as high levels of circulating leptin characterize most cases of human obesity suggesting the development of peripheral leptin resistance (85, 213). Despite the widespread discussion of leptin resistance in obese humans, direct evidence of leptin resistance in peripheral tissues, and more specifically skeletal muscle does not exist. Therefore, the primary purpose of the final two studies of this thesis was to demonstrate the presence of leptin resistance in both rodent and human skeletal muscle. To investigate the development of leptin resistance in rodent skeletal muscle, we based our investigation on a commonly used model of insulin resistance: high-fat feeding. Several studies (194, 205, 321) have previously demonstrated that high-feeding (>45% kcal) with n-6 FA leads to the development of insulin resistance in as little as 2 days and that substitution of a small portion of the n-6 with n-3 FA derived from fish oil restores insulin sensitivity (323). Therefore, we hypothesized that a similar protocol (60% kcal fat, 4 weeks) would induce leptin resistance as assessed by the blunting of leptin's effects on FA oxidation and esterification, while partial substitution with n-3 FA would restore leptin sensitivity.

In the final study of this thesis, we investigated the effects of acute leptin treatment on human skeletal muscle metabolism and more specifically whether there were

differences in leptin responses between lean and obese humans. We hypothesized that in lean humans, leptin would have similar effects to those observed in rodents (i.e. stimulation of FA oxidation and TG hydrolysis while reducing FA esterification into TG), but that in obese humans these effects would be blunted. To test this hypothesis, we measured FA metabolism using the pulse chase technique and human rectus abdominus muscle strips in the presence of pharmacological levels of leptin.

Chapter 3: Fatty Acid Oxidation and Triacylglycerol Hydrolysis Are Enhanced Following Chronic Leptin Treatment in Rats.

Introduction

Initial studies with *ob/ob* mice demonstrated that leptin caused a rapid reduction in food intake, as well as pronounced effects on insulin sensitivity independent of calorie restriction (144). This suggested that leptin may have significant metabolic effects on peripheral tissues such as skeletal muscle. This has been confirmed in several recent rodent studies, in which leptin has been shown to acutely (< 1 hr) increase FA oxidation (241, 242, 316) and TG hydrolysis (316), while decreasing FA esterification into TG in resting skeletal muscle (241, 242, 316). However, the chronic effects of leptin treatment on skeletal muscle FA metabolism have not been examined to date.

Chronic leptin administration results in the depletion of muscle and pancreatic TG stores (58, 367), but whether this is due to increased rates of TG hydrolysis, or lowered rates of FA esterification has not been addressed. Similarly, whether FA oxidation is altered in skeletal muscle by chronic hyperleptinemia has also not been examined. Reduced intramuscular TG stores have been associated with improved insulin sensitivity in both rats (58, 322) and humans (256, 266). Therefore, it is important to understand how chronic leptin treatment alters concentrations of intramuscular TG as this may have important implications for the correction of insulin resistance.

In this study, we utilized the dual label, pulse-chase technique to investigate the effect of chronic (2 wk), moderate hyperleptinemia on FA metabolism in resting and contracting rat soleus (SOL) muscle. It should be stressed that, unlike previous studies which have examined FA metabolism in isolated muscles in the presence of pharmacological levels of leptin (241, 242, 316), the present experiments did not include leptin in the incubation medium. Therefore, we have assumed that any changes in FA metabolism would be the result of prior chronic exposure to leptin; however, it must be acknowledged that secondary effects, such as a decrease in circulating insulin levels as a result of hyperleptinemia, may also be a factor. We hypothesized that in resting and contracting oxidative skeletal muscle, 2 wks of leptin administration would: 1) increase TG hydrolysis and FA oxidation, and 2) decrease the rate of FA esterification into TG. Furthermore, we hypothesized that leptin would alter FA metabolism through 3) enhanced expression of HSL and 4) increased oxidative capacity, as indicated by citrate synthase (CS) and β -hydroxyacyl-CoA dehydrogenase (β -HAD) activities.

Methods

Animals. Rats were randomly assigned to one of the following groups: ad libitum fed-saline treated (AD-S), pairfed-saline treated (PF-S) or leptin (LEPT) treated. Animals were anesthetized with halothane, and a small incision was made through the skin on the upper region of the back between the scapulae. The mini-osmotic pumps (2ML2, Durect Corporation, Cupertino, CA) were filled with either sterile phosphate-buffered saline (AD-S, PF-S) or murine leptin (donated by Amgen, Thousand Oaks, CA), and inserted through the incision. The incision was closed with a single autoclip. A leptin dosage of 0.5 mg/kg/day was used as this has previously been demonstrated to induce moderate hyperleptinemia (22, 152). Animals were then assigned to individual cages and maintained on a reverse 12:12-h light:dark cycle. Water was freely accessible to all groups. Food intake of Purina rodent chow was ad libitum for both the AD-S and leptin treated animals, while PF-S treated animals were fed the same amount of chow as the leptin treated animals. This was determined by measuring the amount of food remaining and the food spillage. Body mass was monitored over the two week treatment period. The Committee on Animal Care at the Universities of Waterloo and Guelph approved all procedures. Two sets of implantation experiments were run in parallel: 1) one set (n = 6-8 per group) for the determination of blood insulin, leptin and FFA, as well as muscle TG, CS, β -HAD, and HSL, and 2) the other for pulse-chase experiments monitoring soleus FA metabolism (n = 8 per group).

Blood and Tissue Sampling. Blood was collected at the completion of treatments (2 wk) via cardiac puncture, following the excision of SOL muscle as described below. All samples were taken while rats were in the fasted state, between 0900 and 1100, to eliminate variability due to diurnal rhythm. Blood was transferred to microcentrifuge tubes where it was allowed to clot before being centrifuged (12 000 x *g* for 2 min) and the serum collected. Serum leptin and insulin levels were assayed in duplicate using RIA kits specific for rat leptin and insulin (Linco, St. Charles, MO). Fatty acids were assayed using a Wako NEFA kit (Wako Chemical, Richmond, VA) and measured on the spectrophotometer (Beckman DU-70) at 550 nm. Serum glucose levels were determined fluorometrically (Perkin Elmer LS50) (27). Soleus muscle TG content was determined on freeze-dried samples, which were dissected free of all visible connective tissue and blood, as previously outlined (122).

Enzyme measurements. Enzyme activity of CS and β -HAD were measured spectrophotometrically in SOL muscle obtained from a separate set of experiments. Soleus muscle was excised and frozen in tongs pre-cooled in liquid N₂ and stored at -80°C until analyzed. The frozen muscle was homogenized in a 20 mM phosphate-glycerol buffer (pH 7.4) and analyzed for maximal activity at 25°C as previously described (69). Briefly, CS activity was determined by initiating the reaction with oxaloacetate and measuring the production of the

colormetric agent DTNB. β -hydroxyacyl-CoA dehydrogenase activity was determined by initiating the reaction with S-acetylaceto-CoA and measuring the production of NAD^+ .

Protein expression of HSL in SOL was measured via Western blotting using procedures described previously (264). Briefly, muscles were homogenized in 210 mM sucrose, 2 mM EGTA, 40 mM sodium chloride, 30 mM HEPES, 5 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride, pH 7.4. Proteins were precipitated with 0.5 M potassium chloride and 25 mM tetrasodium pyrophosphate and spun down in an ultracentrifuge (Beckman XL-90) at 175,000 g for 75 min at 4°C. Protein pellets were rehomogenized in 10 mM Tris-1.0 mM EDTA, pH 7.4, and SDS (Bio-Rad, Mississauga, ON) was added to bring the final concentration to 5%; centrifugation followed at 1,000 g for 10 min at room temperature. Protein concentration was measured using a BCA protein assay.

One hundred micrograms of muscle protein were separated with the use of standard SDS-PAGE (4% stacking gel, 10% running gel) and transferred to membranes (Immobilon polyvinylidene difluoride, Bio-Rad) by electroblotting at 110 V for 1 h in 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol, pH 8.4. Membranes were blocked overnight with 10% (wt/vol) skim milk powder in Tris-buffered saline, pH 7.5, with 0.1% (vol/vol) Tween 20 (Bio-Rad) and then incubated with polyclonal rabbit anti-HSL (1:5,000; kindly donated by Dr.

F. B. Kraemer, Stanford Univ. Medical Center). Secondary incubation with donkey anti-rabbit IgG-horseradish peroxidase conjugate (Amersham Life Science, Oakville, ON) allowed detection with the use of an enhanced chemiluminescence kit (Amersham Life Science). HSL protein band densities were then quantified by scanning the blots and quantifying the density of the protein bands using appropriate software.

Pulse-Chase Studies

For discussions regarding the development of the pulse-chase technique as well limitations and advantages of the technique please see Appendix 2 and 3. For “Quality Control Experiments” completed to verify the viability of the isolated muscle preparation as well as other experiments completed to verify the effectiveness of the pulse-chase technique in monitoring palmitate oxidation and FA incorporation into the TG lipid pools see Appendix 4.

Pre-incubation (equilibration). Following 2 wks of treatment (leptin or saline), rats were anaesthetised with an intraperitoneal injection of sodium pentobarbital (6mg/100g body wt) and the SOL muscle was carefully dissected into longitudinal strips from tendon to tendon using a 27-gauge needle. Two strips were utilized from each SOL muscle. Tendons were sutured, and the strip removed and mounted in a 7 ml incubation reservoir. The sutures were secured to brass hooks to maintain resting tension in the muscle. Seven ml of warmed (30°C), pregassed (95% O₂ /

5% CO₂, pH=7.4) modified Krebs's Henseleit buffer containing 4% FA free BSA (ICN Biomedicals), 2 mM pyruvate, and 0.5 mM palmitate were immediately added to the incubation reservoir. This was the base buffer used in all experiments. Temperature was maintained at 30°C and the incubation medium was continuously gassed.

Pre-experimental labelling of the intramuscular lipid pools. The pulse-chase procedures used have been described previously (104). Briefly, the pre-incubation buffer was drained and a pulse buffer consisting of the base buffer plus 2 µCi of [9,10- ³H] palmitate (Amersham Life Sciences, Oakville, ON, Canada) was added to the reservoir. Muscles were pulsed with [9,10- ³H] palmitate for 40 min to prelabel all endogenous lipid pools (TG, triacylglycerol; DG, diacylglycerol; PL, phospholipid). The pulse buffer was drained and muscles were washed for 30 min with incubation medium containing no radiolabeled palmitate. At the end of the pulse and wash, one SOL strip from each pair was removed, blotted and weighed, and extracted for endogenous lipids as described below, to determine the incorporation of [9,10- ³H] palmitate.

Experimental Phase (Chase). The remaining muscles were incubated for an additional 45 min with 0.5 µCi/ml of [1-¹⁴C] palmitate (Amersham Life Science) at rest, or while stimulated to contract at 20 tetani/min (150 ms train, 60 Hz, 20-40V). During the 45 min chase phase, exogenous palmitate oxidation and

esterification were monitored by the production of $^{14}\text{CO}_2$ and incorporation of [1- ^{14}C] palmitate into endogenous lipids, respectively. Intramuscular lipid hydrolysis was simultaneously monitored by measuring the decrease in lipid [^3H]-palmitate content while endogenous oxidation was monitored by the production of $^3\text{H}_2\text{O}$.

Extraction of Muscle Lipids. Muscles were placed in 13 ml plastic centrifuge tubes containing 5.0 ml of ice-cold 1:1 chloroform methanol (vol/vol) and homogenized using a polytron (Brinkman Instruments, Mississauga, ON, Canada). Following homogenization, connective tissue was removed, weighed and subtracted from the total wet weight. Samples were then centrifuged at $2000 \times g$ (4°C) for 10 min. The supernatant was removed with a glass Pasteur pipette and transferred to a clean centrifuge tube. Distilled water (2.0 ml) was added, samples were shaken for 10 min. and centrifuged as before to separate the aqueous and lipophilic phases. One ml of the aqueous phase was quantified by liquid scintillation counting to determine the amount of ^{14}C labelled oxidative intermediates resulting from isotopic fixation. This represented a 2-fold correction factor for exogenous ^{14}C -palmitate oxidation, as previously described (104, 303).

The chloroform phase, which contains the total lipids extracted from muscle, was gently evaporated under a stream of N_2 and redissolved in 100 μl of 2:1 chloroform-methanol. A small amount of phosphatidylcholine, dipalmitin and tripalmitin (Sigma Chemical, St. Louis, MO, USA) were added to the 2:1

chloroform-methanol to facilitate the identification of lipid bands on the silica gel plates. Fifty μ l of each sample was spotted on an oven-dried silica gel plate (Fisher Scientific Canada, Mississauga, ON). Silica gel plates were placed in a sealed tank containing solvent (60:40:3, heptane - isopropyl ether - acetic acid) for 40 min. Plates were then permitted to dry, sprayed with dichlorofluorescein dye (0.02% w/v in ethanol) and visualized under long-wave ultraviolet light. The individual lipid bands were marked on the plate with a scalpel and scraped into vials for liquid scintillation counting.

Measurement of Endogenous and Exogenous Oxidation. Tritiated H_2O produced from the endogenous oxidation of [9,10- ^3H] palmitate was separated from the labelled substrate by transferring a 1.0 ml of the chase incubation medium to a plastic centrifuge tube containing 5.0 ml of 2:1 chloroform methanol (vol/vol). Samples were then shaken for 10 min before adding 2.0 ml of 2M KCl-HCl, shaken again for 10 min and then centrifuged at 2 000 x g at 4°C for 15 min. A 0.5 ml aliquot was removed from the aqueous phase and quantified by liquid scintillation counting.

Gaseous $^{14}\text{CO}_2$ produced from the exogenous oxidation of [1- ^{14}C] palmitate during the incubation was measured by transferring 1.0 ml of the chase incubation medium to a 20 ml glass scintillation vial containing 1.0 ml of 1 M H_2SO_4 and a 0.5 ml Fisher microcentrifuge tube containing 1 M benzethonium hydroxide.

Liberated $^{14}\text{CO}_2$ was trapped in the benzethonium hydroxide over 60 min. and the microcentrifuge tube containing trapped $^{14}\text{CO}_2$ was placed in a scintillation vial and counted.

Calculations and Statistics. The quantity of palmitate esterified and oxidized was calculated from the specific activity of labelled palmitate in the incubation medium (i.e. radiolabeled palmitate in dpm / total palmitate in nmol). Hydrolysis of intramuscular lipids at rest was calculated from the loss of preloaded [^3H] palmitate (in nanomoles/g wet weight) from each pool. We have previously documented that, during contraction at 20 tetani/min. the loss of each nanomole of incorporated [^3H] palmitate represents ~145 nmol of total FA from the TG pool (102). This ratio was used to calculate the actual rate of net TG hydrolysis during muscle contraction.

All data are reported as mean $\pm$ SE. Results were analyzed using analysis of variance (ANOVA) procedures, and a Tukey's post-hoc test was used to test significant differences revealed by the ANOVA. Significance was accepted at $p \leq 0.05$.

Results

Effects of Chronic Leptin Treatment

Body Composition and Food Intake. Food intakes were significantly reduced in leptin treated animals compared to AD-S (-33%, $p<0.01$; Table 2). Food intake in PF-S animals was matched with leptin treated animals. Food intake was constant over the 2 wk treatment period in all groups. Body mass was reduced in both leptin and PF-S treated animals (-12.5%, $p=0.01$) following 2 wks of treatment (Table 2).

Serum. Relative to AD-S, serum leptin was elevated in leptin treated animals (+418%, $p<0.001$), while being reduced (-73%, $p<0.05$) in the PF-S group (Fig. 2A). Serum levels of insulin and FA (Table 3) were significantly reduced (-85%, $p<0.001$ and -49%, $p=0.05$, respectively) by chronic leptin treatment compared to AD-S. Insulin and FA levels of PF-S animals were unchanged. Glucose levels remained unchanged in leptin and PF-S treated animals (Table 3).

Muscle. Intramuscular TG in SOL was significantly reduced in LEPT animals relative to AD-S (-41%, $p=0.03$) and PF-S (-33%, $p=0.05$) groups (Fig. 2B). Intramuscular TG from PF-S animals was not significantly different from AD-S. There was a significant inverse correlation between serum leptin and intramuscular TG content ($r=0.71$, $p<0.01$; Fig. 2C).

Enzyme Activity. Despite large increases in lipid oxidation, (see below) chronic leptin treatment had no effect on the maximal activity of CS or β -HAD (Table 4). Hormone sensitive lipase was detected in SOL muscle by the identification of a band in the 84-kDa region as previously reported (264). Hormone sensitive lipase expression was also unaltered following chronic leptin treatment (Fig. 3; Table 4).

Metabolic Responses to Chronic Leptin Treatment

Exogenous Palmitate Oxidation and Esterification in Soleus. Chronic leptin treatment had no effect on exogenous palmitate oxidation (Fig 4) in resting SOL muscle. However, during contraction, palmitate oxidation was significantly greater in LEPT animals relative to both AD-S and PF-S animals (Fig 4). Prior exposure to leptin reduced TG esterification at rest (relative to PF-S animals), but not in contracting muscle (Table 5). Esterification of palmitate into the PL and DG pools was also unaffected by leptin (Table 5). Total FA uptake (PL + DG + TG + oxidation; Fig 5) tended to be reduced by leptin at rest (-25%) and during contraction (-7%) relative to PF-S animals, but these differences were not significant. Conversely, there was a trend towards increased total FA uptake in both resting (+18.5%, $p=0.220$) and contracting (+29%, $p=0.064$) muscle from PF-S treated rats, compared to the ad libitum fed group (Fig 5).

Intramuscular Lipid Hydrolysis. Chronic leptin treatment significantly enhanced the rate of TG hydrolysis in both resting ($p<0.001$) and contracting ($p<0.001$) SOL

muscle (Fig. 6). Hydrolysis of the PL and DG pools was negligible in PL and DG pools (i.e. < 5 nmol/g/45 min) and was generally unaffected by leptin treatment (data not shown).

Table 2. Body mass and food intake before and after 14 days of the treatment period.

Parameter	Day 0			Day 14		
	AD-S	PF-S	Leptin	AD-S	PF-S	Leptin
Body Mass, g	248 ± 3	245 ± 6	248 ± 4	267 ± 3	233 ± 6 ^a	234 ± 6 ^a
Food Intake, g/day	18.3 ± 0.5	19.0 ± 0.3	17.5 ± 0.6	17.3 ± 0.5	11.5 ± 0.2 ^a	11.7 ± 0.4 ^a

Values are means ± SE; *n*=12 animals per treatment. ^a Significantly different from AD-S

Table 3. Serum insulin, FA and glucose following 14 days of the treatment period.

Parameter	Treatment		
	AD-S	PF-S	Leptin
Insulin (ng/ml)	1.36 ± 0.03	1.07 ± 0.30	0.18 ± 0.04 ^{ab}
FA (mmol)	0.34 ± 0.04	0.38 ± 0.11	0.17 ± 0.02 ^{ab}
Glucose (mmol)	5.20 ± 0.21	5.14 ± 0.41	5.04 ± 0.48

Values are means ± SE; *n*=12 animals per treatment. ^a Significantly different from AD-S; ^b significantly different from PF-S.

Table 4. Enzyme activity of CS and β -HAD in SOL measured at 24^BC. Hormone sensitive lipase protein expression in SOL.

Enzyme	Treatment		
	AD-S	PF-S	Leptin
CS	23.3 $\pm$ 0.4	23.5 $\pm$ 0.5	23.8 $\pm$ 0.7
β -HAD	3.1 $\pm$ 0.4	3.1 $\pm$ 0.3	2.8 $\pm$ 0.2
HSL	100 $\pm$ 11.0	88.2 $\pm$ 11.7	93.3 $\pm$ 9.7

CS and β -HAD values are means $\pm$ SE and are reported in mmol/min/g wet muscle; $n=12$. HSL protein expression expressed as percentage of AD-S HSL protein density; $n=8$.

Table 5: [¹⁴C] palmitate esterification to TG, DG and PL pools over 45 min in resting and contracting (20 tetani/min) SOL.

Lipid Pool	Rest			Contraction		
	AD-S	PF-S	Leptin	AD-S	PF-S	Leptin
TG	50.9 $\pm$ 5.3	58.5 $\pm$ 4.5	41.8 $\pm$ 4.4 ^b	77.0 $\pm$ 5.9 ^c	90.5 $\pm$ 12.8 ^c	75.7 $\pm$ 9.6 ^c
DG	6.6 $\pm$ 0.8	7.0 $\pm$ 0.3	5.4 $\pm$ 0.8	7.4 $\pm$ 0.7	8.1 $\pm$ 0.8	7.2 $\pm$ 0.9
PL	12.0 $\pm$ 1.2	14.3 $\pm$ 1.8	9.4 $\pm$ 1.5	16.1 $\pm$ 2.3 ^c	20.7 $\pm$ 3.8 ^c	15.5 $\pm$ 1.8 ^c

Values are means $\pm$ SE and are reported as nmol palmitate/g wet wt; $n=8$ experiments. ^aSignificantly different from AD-S; ^bsignificantly different from PF-S; ^csignificantly different from rest.

Figure 2. (A) Serum leptin concentrations; (B) SOL intramuscular TG; and (C) linear regression of serum leptin vs. intramuscular TG after 2 wk treatment period. ^aSignificantly different from AD-S; ^bsignificantly different from PF-S

Figure 3. Representative hormone sensitive lipase Western blot in SOL muscle

Figure 4. Exogenous palmitate oxidation at rest and during contraction (20 tetani/min). Values are means $\pm$ SE, $n=8$. a Significantly different from AD-S; b significantly different from PF-S; c significantly different from rest.

Figure 5. Total FA uptake at rest and during contraction (20 tetani/min). Values are means $\pm$ SE, $n=8$. ^b significantly different from PF-S; ^c significantly different from rest.

Figure 6. Triacylglycerol hydrolysis at rest and during contraction (20 tetani/min). Values are means $\pm$ SE, $n=8$. a Significantly different from AD-S; b significantly different from PF-S; c significantly different from rest

Discussion

Previous studies (58, 367) have demonstrated that chronic leptin treatment reduces TG levels in oxidative rodent skeletal muscle and pancreatic islets, but the mechanism(s) by which this occurs is unknown. In this study, we utilized an isolated skeletal muscle preparation to assess changes in FA metabolism as a result of chronic, *in vivo*, leptin treatment at a physiological concentration. This study differs from previous work by us (316) and others (241, 242) that have demonstrated an acute stimulatory effect on FA metabolism in the presence of pharmacological levels of leptin. It is important to stress that in the present study, FA metabolism was actually monitored in the absence of leptin in the incubation medium, following the 2 wk period of chronic hyperleptinemia. Thus, the observed alterations in FA metabolism are a consequence of prior leptin treatment, and not due to an acute effect. Since chronic exposure to leptin results in a reduction of plasma insulin, we must acknowledge the possibility that our observed changes in muscle FA metabolism may be a secondary effect due to reduced insulin, as opposed to a primary effect due to increased leptin. However, it is our belief that the increase in leptin is physiologically more important, and have discussed this aspect in greater detail below.

Chronic leptin treatment was found to: 1) reduce intramuscular TG stores *in vivo*; 2) increase TG hydrolysis at rest and during contraction; and 3) increase palmitate oxidation during contraction. Citrate synthase and β -HAD activity, as well as HSL expression were unaltered following 2 wk of leptin treatment, suggesting that increased rates of FA oxidation may be due to increased CPT-1 activity, while increased TG hydrolysis is

likely the result of covalent activation of HSL (i.e. phosphorylation). This is the first study to directly demonstrate that chronic leptin treatment enhances FA oxidation and TG hydrolysis in oxidative skeletal muscle, which may explain leptin's ability to reduce intramuscular TG.

Effects of Chronic Leptin Treatment on Serum and Muscle Parameters

Several studies have examined the effects of chronically elevated leptin levels, using either mini-osmotic pumps (21, 22, 70, 350) as in the present study, or via injection of recombinant adenovirus containing leptin cDNA (58). In this study, we obtained moderate levels of hyperleptinemia (~5 fold increase), a level that is similar to that obtained with high-fat feeding in rodents (316). Therefore, unlike numerous other studies that have used pharmacological levels of leptin (70, 350, 367), the levels used in this study are within physiological limits.

Serum FA. One of the characteristics of obesity is the presence of high circulating FA. We (present study) and others (70) have demonstrated that chronic leptin treatment (12-14 days) results in a significant reduction in circulating FA. This may be due to one of two possible mechanisms: a) selective depletion of the labile visceral adipose stores (22), or b) retention of the FA within the adipocyte for oxidation as a result of increased activity of CPT-1, acyl CoA oxidase, and UCP-1 expression in this tissue (84, 288, 289).

Serum Insulin. Acutely, leptin reduces insulin secretion in isolated pancreatic islet cells (63, 369). In this study, chronic leptin treatment reduced serum insulin. However, circulating glucose levels did not become elevated as a consequence, which suggests that insulin sensitivity may have improved. This has been demonstrated in several studies (22, 58, 70, 350, 366, 367), and may be due to the leptin-induced decrease in intramuscular TG (367). The relationship between elevated intramuscular TG and impaired insulin sensitivity has been demonstrated in rodents (58, 322) and humans (256, 266), although the underlying mechanisms are unknown.

Effect of Chronic Leptin Treatment on Lipid Metabolism in Skeletal Muscle. The first site of regulation of FA metabolism in muscle is transport across the sarcolemma, mediated by FA translocase (FAT/CD36) and plasma membrane FA binding protein (FABPpm) (46). FAT/CD36 expression can be altered by perturbations in metabolism such as chronic muscle stimulation (43) and obesity (210). Bonen et al (45) have recently demonstrated that the expression of FAT/CD36 can also be regulated acutely through translocation of the transporter from intracellular stores to the plasma membrane during contraction. In this study, there was a trend towards chronic leptin treatment reducing total FA uptake (esterification + exogenous oxidation) in resting skeletal muscle. This is in agreement with our recent observations that chronic leptin administration reduces both FAT/CD36 and FABPpm content in the sarcolemma (317). However, this effect is nearly eliminated during contraction, supporting our observation that FAT/CD36 is translocated from intracellular compartments to the plasma membrane by contraction, thus increasing

FA uptake (45).

Contrary to the finding observed in leptin treated animals, PF-S treated rats that were calorically restricted by 33% over the 2 wk treatment period, displayed a trend towards increased FA uptake under both resting (+18.5%; $p=0.22$) and contracting (+29%; $p=0.064$) conditions. This finding is in agreement with work by Turcotte et al. (337) demonstrating increased FABPpm content in red skeletal muscle following 48 h of fasting. It is tempting to speculate that the lower serum leptin level during caloric restriction is responsible for an increase in the expression of the FA transporters, resulting in an elevated FA uptake. However, there are clearly numerous hormonal changes that occur during fasting, making it impossible to definitively comment on the isolated effects of reduced leptin levels.

In this study, leptin increased exogenous FA oxidation by >200% in contracting SOL muscle. However, in resting isolated SOL muscle there was no observable chronic effect of leptin on FA oxidation. At rest, the metabolic demands of this quiescent muscle are low due to the lack of innervation and hormonal stimulation. Therefore, it may be necessary to increase the metabolic rate of the SOL in order to detect differences in FA metabolism. Dyck et al. (103) have previously demonstrated similar results in endurance-trained rat SOL muscle, in which the effects of endurance training on FA oxidation were not detected until the metabolic rate was increased with contraction.

The mechanism by which chronic leptin treatment increases FA oxidation in skeletal

muscle is unknown. We hypothesized that leptin would enhance the oxidative potential of skeletal muscle by increasing the activity of key oxidative enzymes, CS and β -HAD. Leptin did not alter the activity of these enzymes, demonstrating that increases in FA metabolism are not due to increased oxidative enzyme capacity. A previous study (152) has also failed to demonstrate any change in CS and β -HAD activity in lean mice following moderate hyperleptinemia. Alternatively, leptin might enhance FA flux into the mitochondrion by a) increasing the expression of CPT-I, or b) by upregulating its activity, possibly through a reduction in ACC2, and consequently, malonyl-CoA. Evidence for these effects has been demonstrated in pancreatic tissue (373), but not skeletal muscle. It is also possible that leptin may increase skeletal muscle metabolic rate and FA oxidation through the expression of the UCP-3 (206). Clearly, more research is required to understand the mechanisms, both acutely and chronically, by which leptin enhanced muscle FA metabolism.

Previously, we have shown that leptin acutely increases TG hydrolysis (316). In this set of experiments we demonstrate that lipolysis is also enhanced following chronic leptin treatment, in both resting and contracting SOL. This increase in TG hydrolysis, determined in isolated SOL, is in agreement with the reduced intramuscular TG content measured in SOL muscle *in vivo*. The enzymatic regulation of TG breakdown in muscle is poorly understood. It is believed that the neutral lipase, HSL, is the key rate-limiting enzyme for regulating intramuscular TG breakdown (343). We measured total HSL protein expression and found that leptin had no effect on total protein expression.

Langfort et al. (196) has previously demonstrated that epinephrine activates HSL by phosphorylation, without affecting total enzyme concentration. Thus, it is possible that leptin also increases HSL activity by altering its state of phosphorylation.

While the elevation of serum leptin was associated with numerous significant changes in muscle FA metabolism, we generally failed to note any significant changes in FA metabolism in PF-S animals, despite the fact that serum leptin was significantly decreased in this group. However, trends towards increased oxidation and total FA uptake were noted in this group. Although we cannot provide definitive reasons for the absence of significant effects, other factors may have countered the effects of a lower leptin concentration, including other hormonal changes (e.g. increased catecholamines, cortisol), or an altered sensitivity to leptin.

Potential Role of Insulin. It has generally been assumed that alterations in metabolism as a consequence of chronic leptin exposure are due directly to the elevated levels of leptin. However, as confirmed in the present study, chronic leptin administration, in the absence of other perturbation (such as diet), may result in the lowering of plasma insulin (152, 300). Since insulin has been shown to stimulate FA uptake and esterification, and decrease oxidation and hydrolysis in skeletal muscle (10, 105, 242), it cannot be discounted that a decrease in insulin is at least partly responsible for the observed changes in muscle metabolism. Thus, it could be argued that changes in muscle FA metabolism are a secondary, and not a primary effect of leptin. However, there are two

reasons why we feel that the observed metabolic changes are a primary effect of leptin. Firstly, the absolute magnitude of change in leptin (from 1.5 (AD-S) to 9 ng/ml (LEPT)) was much greater than the absolute change in insulin (from 1.36 to 0.18 ng/ml) in the present study. Secondly, in a recent study by Yaspelkis (367), the injection of leptin for 12 to 15 d significantly lowered muscle TG and improved insulin responsiveness in rats fed a high-fat diet, despite the fact that insulin levels were not significantly lowered. Clearly, without knowing the differences in sensitivity of muscle to insulin and leptin, or the threshold concentrations for eliciting a chronic effect, we cannot unequivocally say that the decrease in insulin was without effect.

Summary. We have demonstrated that chronically elevated levels of serum leptin may reduce intramuscular TG in lean rats by stimulating FA oxidation and TG hydrolysis, and by reducing total FA uptake. Thus, leptin is an important factor in regulating intramuscular TG content *in vivo*. However, in human obesity, intramuscular TG is elevated, despite high levels of circulating leptin (85). We speculate that, in obesity, the development of leptin resistance leads to decreased FA oxidation and TG hydrolysis, resulting in the accumulation of intramuscular TG stores and development of insulin resistance. Research examining the effects of leptin in lean and obese human skeletal muscle FA metabolism is needed.

Chapter 4: Chronic Leptin Administration Decreases Fatty Acid Uptake and Fatty Acid Transporters in Rat Skeletal Muscle

Introduction

The development of obesity and insulin resistance in both humans and rodents is associated with abnormalities in lipid metabolism, involving impaired FA oxidation and increased storage as intramuscular TG (256, 267). While the association of insulin resistance with increased concentrations of intramuscular TG is well recognized (58, 256, 267), the underlying mechanisms are unknown. In *ob/ob* mice, the absence of leptin results in a phenotype characterized by obesity and insulin resistance, and treatment with recombinant leptin results in a rapid reduction in body adiposity and the restoration of insulin sensitivity (260). In skeletal muscle, an essential tissue responsible for regulating whole body lipid and glucose metabolism, leptin has been shown to increase FA oxidation and intramuscular TG hydrolysis acutely (<1 h) (241, 242, 316) while decreasing FA esterification (316).

The effects of chronic (>7 days) leptin treatment on skeletal muscle FA uptake have not been examined. Several studies have demonstrated that chronic leptin treatment in lean and diabetic rats, as well as *ob/ob* mice, leads to reduced body mass, and results in significant reductions in circulating insulin, independent of reduced food intake (70, 152, 350), suggesting an improved insulin sensitivity. Chronic hyperleptinemia has also been shown to increase glucose uptake in skeletal muscle (58, 70). However, this is not due to increased GLUT-4 expression, suggesting that leptin may be altering the transporter's

intrinsic activity and/or translocation to the sarcolemma (350). Another possible mechanism for improved insulin sensitivity may be the decrease of intramuscular TGs observed in red skeletal muscle following leptin treatment (58), since intramuscular TG accumulation is correlated with insulin resistance (58, 256, 267). Thus, it is important to understand the mechanisms by which chronic leptin treatment alters intramuscular TG concentrations. A possible mechanism contributing to the insulin-induced reductions in intramuscular TGs may occur at the level of FA transport into the muscle cell.

Fatty acids traverse the plasma membrane via passive diffusion and a protein-mediated mechanism (46). Several proteins have been shown to facilitate FA transport, including the fatty acid translocase (FAT/CD36) and plasma membrane associated fatty acid binding protein (FABPpm) (46). While fatty acid transport protein 1 (FATP1) was initially thought to be a FA transporter (290), recent studies have shown that FATP1 is a very long chain fatty acyl-CoA synthetase (79). The cytosolic, 15 kDa fatty acid binding protein (FABPc) is also an important feature of the FA transport system, since it acts as a FA sink once FAs have crossed the plasma membrane (46).

In muscle, FABPc is present in great excess and therefore does not limit FA uptake (46). However, increasing the expression of FAT/CD36 in skeletal muscle increases FA transport and oxidation (169), whereas in FAT/CD36 null mice the uptake of FAs is reduced (116). Other mechanisms besides altered FAT/CD36 expression can also regulate FA transport. In contracting muscles, FAT/CD36 is translocated, within

minutes, from an intracellular pool to the plasma membrane, resulting in an increased rate of FA transport (45). Thus, skeletal muscle FA transport can be affected in a number of ways, by altering the expression of FAT/CD36 and/or relocating this protein to the plasma membrane.

A link between leptin-induced reductions in intramuscular TG depots and improved insulin sensitivity in skeletal muscle may be the reduced uptake of FAs into the muscle cell. Fatty acids are known to induce insulin resistance in muscle (39), and limiting their entry into the muscle cell may be expected to reduce FAs and FA intermediate accumulation in the cytosol of muscle, thereby improving insulin sensitivity. We, therefore, hypothesized that chronic leptin treatment can lead to a reduced rate of FA transport into muscle due to reductions in skeletal muscle FA transporters, FAT/CD36 and FABPpm. We have examined the effects of chronic hyperleptinemia (2 weeks) on FA transport, the expression of FA transporters (FAT/CD36 and FABPpm) as well as their localization in the plasma membrane. The present studies have shown that leptin treatment (2 weeks) repressed FAT/CD36 expression in muscle, and reduced plasma membrane FAT/CD36 and FABPpm, which resulted in a reduced FA transport across the sarcolemmal membrane.

Methods

Animals. Female Sprague-Dawley rats (247.6 ± 2.6 g) were randomly assigned to one of three groups (ad libitum fed-saline treated (control), pairfed-saline treated (pairfed) or leptin treated, $n=8$ per group). In anesthetized (halothane) animals mini-osmotic pumps (2ML2, Durect Corporation, Cupertino, CA) were implanted subcutaneously, slightly posterior to the scapulae. Pumps were filled with either sterile, phosphate-buffered saline (control, pairfed) or leptin (Amgen, Thousand Oaks, CA). A leptin dosage of 0.5 mg/kg/day was used as this had been previously demonstrated to induce moderate hyperleptinemia (22, 152). Animals, assigned to individual cages, were kept on a reverse 12:12-h light:dark cycle. Water was freely accessible for all groups. Food intake of Purina rodent chow was ad libitum for both the control and leptin treated animals while pairfed treated animals were fed the same amount of chow as the leptin treated animals consumed. Body mass was monitored weekly over the 2 wk treatment period. The committees on Animal Care at the Universities of Waterloo and Guelph approved all procedures.

Blood and Tissue Sampling. Blood samples were collected at the completion of treatment (2 wk) via cardiac puncture after excision of red and white skeletal muscle. Samples were taken in the fasted state between 0900 and 1100 to eliminate diurnal variability. Serum leptin and insulin concentrations were assayed in duplicate using RIA kits (Linco, St. Charles, MO) specific for rat leptin and insulin. Fatty acids were assayed using a Wako NEFA kit (Wako Chemical, Richmond, VA). Serum glucose levels were

determined fluorometrically (27). Soleus muscle intramuscular TG content was determined on freeze-dried samples, which were dissected free of all visible connective tissue and blood, as previously outlined (122).

Giant Sarcolemmal Vesicles. Vesicles from red (vastus intermedius, red vastus lateralis, red gastrocnemius, red tibialis anterior) and white muscles (plantaris, white vastus lateralis, white gastrocnemius, white tibialis anterior) were prepared, as we have described in detail previously (46). Soleus muscle was not used because this muscle is not large enough to make giant sarcolemmal vesicles and measure the mRNA and protein content of the fatty acid transporters. Briefly, rat hindlimb muscles from both legs were divided into pools of red muscles (vastus intermedius, red vastus lateralis, red gastrocnemius, red tibialis anterior) and white muscles (plantaris, white vastus lateralis, white gastrocnemius, white tibialis anterior) on the basis of the fiber composition. The muscles samples were cut into thin layers (~1-3 mm thick) and incubated for 1 h at 34°C in 140 mM KCl-10mM MOPS (pH 7.4), collagenase (150 U/ml), and aprotinin (0.01 g/ml). The muscle was then washed with KCl/MOPS and 10mM EDTA, and the supernatant was collected. Percoll (final concentration 16%) and aprotinin were added to the supernatant. This supernatant was placed at the bottom of a density gradient consisting of a 3-ml middle layer of 4% Nycodenz (wt/vol) and a 1-ml KCl-MOPS upper layer. The samples were spun at 60 g for 45 min at room temperature. After centrifugation, the vesicles were harvested from the interface of the two upper solutions. The vesicles were diluted in KCl-MOPS and recentrifuged at 800 g for 30 min. Vesicles

were immediately used for transport experiments. In addition, some of the vesicles were placed in a blood cell counting chamber and were photographed under a phase contrast microscope to determine vesicle size and density. Remaining vesicles were stored at -80°C for determination of plasma membrane FAT/CD36 and FABPpm.

Fatty Acid Transport. Palmitate uptake was measured by addition of unlabeled palmitate (15 μ M) and radiolabeled ^3H palmitate (0.3 μ Ci, Amersham) and ^{14}C mannitol (0.06 μ Ci, Amersham) in a 0.1% BSA-KCl-MOPS solution to 40 μ l of vesicles (80 μ g protein). The reaction was carried out at room temperature for 15-s. Palmitate uptake was terminated by addition of 1.4 ml ice-cold KCl-MOPS, 2.5 mM HgCl₂, and 0.1% BSA. The sample was quickly centrifuged at maximal speed in a microfuge for 1 min. The supernatant was discarded and radioactivity was measured by adding the stop solution to the membrane before the addition of the isotopes. To calculate palmitate transport, the contribution of palmitate diffusion was subtracted from the palmitate uptake as has previously been used for determining lactate (225) and FA transport (46).

Western Blotting. The putative FA transporters FAT/CD36 and FABPpm were measured in muscle homogenates (from 50-80 mg wet wt. of red and white gastrocnemius muscle which was excised quickly and frozen in liquid N₂) as well as in plasma membranes of giant sarcolemmal vesicles. Muscles were homogenized in 210 mM sucrose, 2 mM EGTA, 40 mM sodium chloride, 30 mM HEPES, 5 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride, pH 7.4. Proteins were precipitated with 0.5 M potassium chloride and 25 mM tetrasodium pyrophosphate and spun down in an ultracentrifuge

(Beckman XL-90) at 175,000 g for 75 min at 4°C. Protein pellets were rehomogenized in 10 mM Tris-1.0 mM EDTA, pH 7.4, and SDS (Bio-Rad, Mississauga, ON) was added to bring the final concentration to 5%; centrifugation followed at 1,000 g for 10 min at room temperature. Protein concentration was measured using the BCA protein assay.

To detect FAT/CD36, we used the monoclonal antibody MO-25 (Narendra N. Tandon, Thrombosis Research Laboratory, Otsuka Maryland Research Institute, Rockville, Maryland) that has been used in previous studies to detect CD36 (45, 220). To detect FABPpm, we used an antibody prepared by J. Calles-Escandon (Glaxo Smith Kline, Miami, Florida). Western blot procedures were as described previously (46). Briefly, plasma membranes (10 µg protein) or muscle homogenates (25 µg protein), with prestained molecular weight markers (Bio-Rad) were separated on 12% SDS-polyacrylamide gels (150 V for 1 h). Proteins were then transferred from the gel to Immobilon polyvinylidene difluoride membranes (25 V, 25 min). Membranes were then shaken for 1 h at room temperature in *buffer A* [20 mM Tris . base, 137 mM NaCl, 0.1 M HCl (adjusted to pH 7.5), 0.1% (vol/vol) Tween 20, and 10% (wt/vol) non fat dried milk (FABPpm) or 5% (wt/vol) BSA (FAT/CD36)]. Membranes were then incubated at room temperature on a shaker for 1 h in primary antibody which contained the antibody for FAT/CD36 (1: 10000, vol/vol) or FABPpm (1 : 5000 vol/vol) in *buffer A* (as described above). Membranes were then washed twice for 15 and 5 min in *buffer B* (*buffer A* without dried milk or BSA) followed by incubation for 1 h with secondary antibodies for FAT/CD36 (1: 20,000 with donkey anti mouse immunoglobulin G horseradish peroxidase)

and FABPpm (1: 3000 with donkey anti rabbit immunoglobulin G horseradish peroxidase) in *buffer B*. Membranes were then washed in *buffer B* once for 15 min and four times for 3 min. FAT/CD36 and FABPpm were then detected using an enhanced chemiluminescence detection method by exposing the membranes to film (Hyperfilm-ECL) at room temperature according to the instructions of the manufacturer. Film was developed and fixed in GBX fixer/replenisher (Kodak). FABPpm and FAT/CD36 protein band densities were then quantified by scanning the blots and quantifying the density of the protein bands using appropriate software.

RNA Isolation. Messenger RNA for FAT and FABPpm were measured in red and white vastus muscle using procedures described previously (103). Total RNA was isolated from 100-150 mg wet wt. of red and white gastrocnemius muscle (which was excised quickly and frozen in liquid N₂) by using the guanidine isothiocyanate/cesium chloride centrifugation method (71) with minor modifications. The tissues were homogenized in 10 ml of 4 M guanidine isothiocyanate and layered on top of 3.3 ml of 5.7 M of cesium chloride solution. The samples were centrifuged in an SW-41 Ti rotor (Beckman Canada, Mississauga, ON, Canada) at 30,000 rpm for 23 h. The RNA pellets were recovered and purified by two precipitations in ethanol.

Northern Blot Analysis. Five micrograms of total RNA were used for electrophoresis on 1.2% formaldehyde agarose gels and then transferred to positively charged nylon membrane (Boehringer Mannheim, Laval, QC, Canada). RNA integrity was determined

on a 1% formaldehyde denaturing gel, separated on an agarose gel, and transferred overnight to an uncharged nylon membrane. To check that RNA (10 µg/lane) was intact and evenly loaded and to check transfer to the nylon membrane, RNA was stained with methylene blue to visualize 28S and 18S ribosomal bands (corrections for total RNA loading errors were made using the 18S signal RNA). After prehybridization of the membrane for at least 4 h at 68°C, the DIG Easy-Hyb hybridization buffer was replaced with buffer containing DIG-labelled antisense RNA probe and the membrane was incubated with the cDNAs for FAT and FABPpm probes overnight at 68°C. The Northern blots were then ultraviolet-cross-linked with a GS-Gene linker (Bio-Rad, Hercules, CA). Chemiluminescent detection was performed in accordance with the protocol supplied by the manufacturer (Boehringer Mannheim), and the membrane was exposed to Kodak BioMax film. After exposure, the film was developed in Kodak developer and fixed in Kodak fixer.

Results

Body Composition and Food Intake. Food intakes were significantly reduced in leptin treated animals (-33%, $p < 0.01$) compared to ad libitum fed controls (Fig. 7A). In pairfed treated animals, food intake was matched with leptin treated animals. Over the 2-week treatment period, food intake was constant in all groups. Body mass was reduced in both leptin and pairfed treated animals (-12.5%, $p < 0.05$) compared to controls following two weeks of treatment (Fig. 7B).

Circulating Concentrations of Leptin, Insulin, Glucose and Fatty Acids. Chronic leptin treatment increased circulating leptin (8.75 ± 0.75 ng/ml) compared to control (1.72 ± 0.30 ng/ml) and pairfed animals (0.5 ± 0.10 ng/ml) ($P < 0.05$). In contrast, leptin treatment reduced circulating insulin (0.20 ± 0.05 ng/ml) and FAs (0.18 ± 0.04 mM) compared to control (insulin 1.30 ± 0.04 , FA 0.35 ± 0.05) and pairfed animals (insulin 1.17 ± 0.30 ng/ml, FAs 0.38 ± 0.11 mM) ($P < 0.05$). Glucose concentration did not differ among the three groups of animals (5.05-5.20 mM) ($P > 0.05$).

Intramuscular Triacylglycerols. Intramuscular TG (Fig. 8) in soleus muscle was significantly reduced in leptin treated animals, relative to control (-41%, $p = 0.03$) and pairfed animals (-33%, $p = 0.05$). Intramuscular TGs of pairfed animals was not significantly different from controls.

Figure 7. Daily food consumption of animals during a 2 week period (A) and change in body mass of animals during a 2 week period (B). Values are means $\pm$ SE, $n=8$. a, significantly different from control; b, significantly different from time 0

Figure 8. Intramuscular triacylglycerol concentrations following a 2 week treatment period. Values are means $\pm$ SE, n=8. a, significantly different from control; b, significantly different from pairfed.

FAT/CD36 and FABPpm mRNA and Protein Expression. With leptin treatment, FAT mRNA abundance was significantly reduced in both red (-70%, $p<0.001$) and white (-48%, $p<0.01$) muscles (Fig 9A), while FABPpm mRNA abundance was unchanged (Fig. 9B). We measured the protein expression of FAT/CD36 and FABPpm in both red and white muscle homogenates (intracellular + plasma membrane pools) as well as in plasma membrane only fractions derived from giant sarcolemmal vesicles. Chronic leptin treatment reduced FAT/CD36 protein in red (-32%, $p<0.01$) but not white muscle homogenates (-15%, $p>0.05$) (Fig.10A). FABPpm protein in both red and white muscle homogenates was unchanged with leptin treatment (Fig. 10B). Plasma membrane FAT/CD36 (Fig. 11A) and FABPpm (Fig. 11B) were significantly reduced following leptin treatment in both red and white muscles (FAT/CD36: red -49%, white -57%; FABPpm: red -26%, white -43%, $P<0.05$).

Palmitate Transport in Giant Sarcolemmal Vesicles. To determine whether leptin affected FA transport in muscle, giant sarcolemmal vesicles obtained from red and white skeletal muscle were used. We have previously characterized FA transport in red and white muscle (46). The giant vesicles from both red and white muscle were spherical in appearance and averaged $13.8 \pm 0.05 \mu\text{m}$ ($n=120$) in diameter, and vesicle size was similar in all groups ($p>0.05$) (See Appendix 4, Figure 27). As previously demonstrated (46) red muscle contained a greater sink for incorporated palmitate due to an elevated FABPc content (red, $1.53 \pm 0.25 \text{ mg/g wet wt}$; white, $0.23 \pm 0.05 \text{ mg/g wet wt}$, $p<0.001$).

There was no difference in FABPc content among treatments. As we have demonstrated previously (46), palmitate uptake was greater in red vs. white skeletal muscle (+58%, $p < 0.001$, Fig. 12). Palmitate uptake was significantly reduced in leptin treated vs. control animals in both red and white skeletal muscle (-33% and -46%, respectively, $p < 0.05$, Fig. 12), but was not different between pairfed and control animals.

Comparison of Fatty Acid Transport and Plasma Membrane FAT/CD36 and FABPpm

We compared the rates of FA transport with the plasma membrane FAT/CD36 and FABPpm. For these purposes, the data from all the experimental groups and red and white muscles were used. These comparisons showed that palmitate uptake by giant sarcolemmal vesicles was highly correlated with the plasma membrane FAT/CD36 protein ($r = 0.88$, $p < 0.01$, Fig. 13A) and plasma membrane FABPpm protein ($r = 0.94$, $p < 0.01$, Fig. 13B).

A

B

Figure 9. mRNA abundance of FAT (A) and FABPpm (B) in red and white vastus muscle following a 2 week treatment period. Values are means $\pm$ SE, $n=8$. a, significantly different from control; b, significantly different from paired; c, significantly different from red.

Figure 10. Muscle homogenate protein expression of FAT/CD36 (A) and FABPpm (B) in red and white gastrocnemius muscle following a 2 week treatment period. Values are means $\pm$ SE, $n=8$. a, significantly different from control; b, significantly different from pairfed; c, significantly different from red.

Figure 11. Plasma membrane protein expression of FAT/CD36 (A) and FABPpm (B) in red and white gastrocnemius muscle following a 2 week treatment period. Values are means $\pm$ SE, $n=8$. a, significantly different from control; b, significantly different from paired.

Figure 12. Fatty acid transport into giant sarcolemmal vesicles derived from red and white gastrocnemius muscle following a 2 week treatment period. Values are means $\pm$ SE, $n=8$. a, significantly different from control; b, significantly different from paired; c, significantly different from red.

Figure 13. Relationship between plasma membrane FAT/CD36 (A) and FABPpm (B) and palmitate transport into giant sarcolemmal vesicles. Data are from Figures 11 and 12. Values are means $\pm$ SE, n=8.

Discussion

The movement of FAs across the sarcolemma, involves the FA transporters FAT/CD36 (3, 28) and FABPpm (372), and is the first step in the regulation of FA metabolism in muscle. Recent studies in our laboratory (43, 46, 103) and others (29, 229) have demonstrated that FA transporter expression is regulated by the metabolic demand of skeletal muscle (43), obesity (29, 229) and diabetes (29). These latter studies (29, 229) suggest that there may be hormonal regulation of FA transporter expression, resulting in altered rates of plasmalemmal FA transport. Leptin may be one of the endocrine signals regulating FA transporter expression, and skeletal muscle may be an important target for leptin. This tissue is important for regulating FA homeostasis, because of its mass (40% of body weight) and highly variable metabolic rate.

In isolated muscles, the acute (≤ 60 min) effects of leptin include an increased rate of FA oxidation and a concomitantly reduced rate of esterification (241, 242, 316). Prolonged hyperleptinemia (6-14 days) reduces muscle TG depots (299), an effect that may be achieved, in part, by reducing the protein-mediated uptake of FAs into the myocyte. Therefore, we have investigated the effects of chronically (14 days) elevated circulating leptin levels on FA transporter expression and localization in red and white rat skeletal muscle, as well as on FA transport into giant sarcolemmal vesicles derived from these two types of muscle. Several novel findings are reported in this study. Leptin treatment 1) reduced FAT mRNA abundance and the expression of FAT/CD36 protein, while FABPpm mRNA and protein expression were not altered, however, 2) both of the FA

transport proteins, FAT/CD36 and FABPpm, located at the plasma membrane were reduced, which 3) resulted in a reduced rate of FA transport into red and white skeletal muscle giant sarcolemmal vesicles. These effects were not observed in pair-fed animals that lost the same body weight as the leptin-treated animals.

Importantly, the chronic (2 weeks) effects of leptin on FA uptake and transporters are not comparable to studies in which isolated muscles have been acutely (≤ 60 min) exposed to leptin (241, 242, 316). In those studies leptin did not alter FA uptake, rather leptin repartitioned the FAs taken up towards oxidation and away from esterification (241, 242, 316).

In this study, we induced moderate levels of hyperleptinemia (~ 4 fold increase), a level that is similar to that obtained following 2 wk of high-fat feeding in rodents (316). This physiologic increase in leptin reduced intramuscular TG depots, and circulating insulin and FAs, while not altering circulating glucose concentrations. These results parallel studies in which pharmacological levels of leptin have been administered (21, 70, 299, 366, 367). Since the serum insulin concentrations were already quite low in the control animals, it seems unlikely that a retarded rate of insulin-stimulated FA esterification, rather than the increased leptin concentrations, accounted for the reduction in the intramuscular TG depots. The reduction in circulating FAs is probably due to a selective depletion of the labile visceral adipose stores (22). Therefore, in the short term (a time point not measured in this study), it would be expected that serum FA levels would be

elevated, but as the fat mass decreases with prolonged leptin treatment, FA levels may become normalized (58, 350, 367) or decrease below normal levels (70).

The repression of FAT/CD36, after 2 weeks of leptin treatment, is likely not attributable to the change in circulating glucose, insulin or FAs. Glucose concentrations were not changed in the leptin treated animals, and based on evidence from other studies in our laboratory, there is also no relationship between the expression of FA transporters and the circulating levels of either insulin or FAs. For example, reductions in circulating insulin, induced by leptin (present study) or severe diabetes (Luiken, unpublished data and (261)), are associated with either a decrease (leptin) or an increase (diabetes) in FA transporters. An increase in circulating insulin, such as observed in obese Zucker rats, does not alter skeletal muscle FA transporter expression (210). A recent report has shown that increasing circulating FAs, 6-fold in excess of the normal physiological range, reduces total FAT/CD36 expression (158). But, in studies in our laboratory we do not observe a relationship between circulating FAs and FAT/CD36 or FABPpm expression. When circulating FAs are increased (severe diabetes, Luiken et al., unpublished data; obese Zucker rats, (210)), there is either no change in FA transporters (obese Zucker rats, (210)) or there is an increase (severe diabetes) (Luiken, unpublished data and (261)). With mild diabetes circulating FAs are not altered, yet FA transporter expression is increased (Luiken et al., unpublished data). Thus, the available evidence suggests that neither, glucose, insulin nor FAs are associated with changes in the expression of FAT/CD36. Therefore, it is appropriate to conclude that the present results are attributable to leptin.

The reduced serum insulin and unaltered glucose concentrations indicated that insulin sensitivity was improved, an observation that has previously been observed following chronic leptin treatment (58, 70, 350, 367). This improved insulin sensitivity is likely due to the reductions in muscle TG depots, since the relationship between elevated intramuscular TG depots and impaired insulin sensitivity is well established in rodents (58, 322) and humans (256, 266), although the underlying mechanism(s) are unknown. It has been suggested that intramuscular TG depots may reduce insulin sensitivity by impairing the insulin-signaling pathway (145, 184). Since FA transport in the leptin treated animals was reduced, this may also contribute to reducing the intramuscular TG depots, and in this manner contribute to the improved insulin sensitivity observed in leptin treated animals.

The giant sarcolemmal vesicle preparation used in our studies allows for a true measurement of FA transport, independent of metabolism (46). Unlike other preparations that have been used to measure FA transport, such as hepatocytes, cardiomyocytes, and adipocytes (29, 229), giant sarcolemmal vesicles provide many advantages. We (46) have shown that in giant sarcolemmal vesicles, (a) initial rates of FA uptake can be determined, (b) giant vesicles contain cytosolic FA binding protein (FABPc) in excess, which provides for a large intravesicular FA sink, (c) all of the palmitate taken up by the vesicles is fully recovered as unesterified palmitate (i.e. none of the palmitate taken up is esterified, oxidized or associated with the plasma membrane).

and (d) vesicles are 100% oriented right side out. Therefore, the giant sarcolemmal vesicle preparation used in the present study provides an appropriate model with which to examine leptin's effects on FA transport in skeletal muscle.

In the present study, we observed that chronic leptin treatment reduces palmitate transport into giant sarcolemmal vesicles. This reduction was associated with concomitant reductions in plasma membrane FAT/CD36 and FABPpm proteins. Previously, we have shown that FA uptake in heart and skeletal muscle is highly correlated with the FA transporters, FAT/CD36 and FABPpm, but not FATP1, located at the plasma membrane (46, 212)). This correlation between these plasmalemmal FA transporters and fatty transport was confirmed in the present studies (Fig 13). It is believed that FABPpm and FAT/CD36 may interact with each other to facilitate FA uptake across the sarcolemma (46), but the specific role of each transporter has not been completely elucidated. It is known that both proteins are critical for mediating FA transport in skeletal muscle, because blocking of either transporter results in significantly reduced rates of FA uptake (212). While chronic leptin treatment led to significant reductions in both FAT/CD36 and FABPpm protein in the plasma membrane of skeletal muscle, the mechanisms by which these reductions occurred were different for the two transport proteins.

The regulation of expression of FAT/CD36 and FABPpm has been examined in only a few studies. At the level of their mRNAs, one or both of these transporters are altered in

some, but not all models of genetic obesity and diabetes, and this seems to depend also on the tissue being examined (28, 229, 372). Altering the metabolic demands of the muscle by chronic muscle contraction for 7 days (43) has been demonstrated to increase the expression of FAT/CD36 and FA transport rates in skeletal muscle. In the present experiments, leptin decreased both the FAT mRNA abundance and the expression of FAT/CD36 protein in red and white skeletal muscles, suggesting that prolonged exposure to leptin reduced the transcription of FAT. Contrary to the effects on FAT/CD36, leptin did not alter the FABPpm mRNA abundance or its protein product. Thus, in muscle, leptin alters the expression of FAT/CD36, but not FABPpm

Our studies demonstrate clearly that whether or not the expression of the FA transport proteins are altered, FA transport can be lowered due to a reduction in plasmalemmal FAT/CD36 and FABPpm. We have recently shown that FAT/CD36 is located both at the plasma membrane and in an intracellular (endosomal) depot (45). Muscle contraction causes a translocation of the FAT/CD36 transporter from endosomal compartments to the plasma membrane within 5 min of the onset of stimulation, leading to an increase in FA transport rates (45). Thus, the plasmalemmal localization of FAT/CD36 can be regulated independently of the total available pool, analogous to the regulation of GLUT-4. However, in the present study the leptin-induced reductions in plasmalemmal FAT/CD36 would seem to be attributable to the reduced expression of this protein and not its intracellular redistribution.

In contrast, the leptin-induced reductions in plasmalemmal FABPpm cannot be explained by reductions in the total pool of this transporter, since the total FABPpm availability was not affected by leptin treatment. This suggests that the localization of FABPpm in the plasma membrane is also an important means to regulate FA uptake. The selective reduction in plasma membrane FABPpm in the face of unaltered total quantities of muscle FABPpm protein content suggests that there may therefore also be an intracellular pool of FABPpm. Indeed, we now have preliminary evidence for this suggestion (Bonen et al. unpublished data).

Dietary and genetic models of rodent obesity and diabetes, are characterized by either a lack of leptin (*ob/ob* mice) (368), or leptin receptor defects (*db/db* mice and obese Zucker (*fa/fa* rat) (72, 331). In skeletal muscles of obese Zucker (*fa/fa*) rats, FAT/CD36 and FABPpm expression are not altered, but there is an increased rate of FA transport, due to an increase in plasmalemmal FAT/CD36 (210). In other studies it has been shown that transcripts of FATCD36, FABPpm and FATP1 are increased in liver and adipose tissue of *ob/ob* and *db/db* mice, and obese Zucker *fa/fa* rats (28, 229, 372). Based on these foregoing studies, it seems plausible that the lack of leptin action leads to the overexpression of FAT/CD36 and FABPpm (28, 229, 372), and/or the subcellular redistribution of FAT/CD36 (210). These effects may be associated with increased rates of FA uptake, with a resultant accumulation of intracellular TG depots in muscle, liver and adipose tissue in these models (28, 229, 372).

We have clearly demonstrated that chronic leptin exposure represses FAT/CD36.

However, this may not occur in obese humans, who characteristically exhibit elevated circulating leptin levels, since in such individuals skeletal muscle FABPpm is increased (305). It may be possible that during the development of human obesity, skeletal muscle becomes resistant to leptin, resulting in the overexpression of the FA transporters leading to the accumulation of intramuscular TG depots. We have previously demonstrated that rodent skeletal muscle becomes resistant to leptin following the consumption of high-fat diets (316). The apparently different responses to leptin in rodents and humans suggests that there can be species differences in the molecular responses to leptin.

A number of studies have shown that leptin reduces body weight, due to a large reduction in fat mass (21, 22). Concomitantly, skeletal muscle intramuscular TG depots are also reduced (58, 367). Presumably, the loss of fat mass indicates an enhanced rate of FA metabolism. Indeed, a number of reports have shown that acute (< 60 min) exposure to leptin augments FA oxidation in isolated skeletal muscle (241, 242, 316). Therefore, it seems somewhat anomalous that with chronic leptin treatment FA transport is reduced. However, a primary function of leptin may be to limit the accumulation of intramuscular TG depots, as has been speculated by others (341). Thus, leptin-stimulated increases in the rates of FA oxidation and TG hydrolysis, along with reduced rates of esterification (241, 242, 316) can be seen as effective mechanisms to lower intramuscular TG depots. Hence, limiting FA entry into the myocyte over the long term may also be part of the strategy to reduce the intramuscular TG depots. These foregoing suggestions require

further study.

The leptin effects on FA transport and transporters were more pronounced in oxidative types of muscle when compared to the more glycolytic types of muscle. Similarly, in CD36 null mice, a greater reduction in FA uptake occurred in oxidative muscles than in glycolytic muscles (78). When we examined the effects of reduced muscle activity (denervation) on the changes in glucose transport and transporters (227, 228), and lactate transport (224) and monocarboxylate transporters (362), the greatest effects were also observed in the more oxidative types of muscles. Thus, it appears that oxidative types of skeletal muscle are more susceptible to alterations in their substrate transport capacities and transporter expression than glycolytic muscles. The basis for this susceptibility is not known.

In conclusion, the present study has demonstrated that chronic leptin treatment reduces circulating insulin and FA levels and decreases the storage of intramuscular TG depots in skeletal muscle. In addition, leptin reduces the content of FAT/CD36 and FABPpm in the plasma membrane of both red and white skeletal muscle, leading to a reduction in FA transport. These plasma membrane reductions in FABPpm occurred in the face of unaltered levels of FABPpm mRNA and FABPpm protein expression, while reduced plasma membrane FAT/CD36 is due to reductions in FAT mRNA and FAT/CD36 protein. Thus, chronic leptin treatment limits the uptake of FAs by skeletal muscle.

Chapter 5: Development of Leptin Resistance in Rat Soleus Muscle Following High-Fat Feeding

Introduction

Leptin, the product of the *ob* gene, is a peptide hormone that is produced by adipose tissue and has been shown to regulate food intake and energy expenditure (373). In *ob/ob* mice, the absence of leptin results in a phenotype characterized by obesity and insulin resistance (260). Daily injection with exogenous recombinant leptin results in a rapid reduction in food intake, body adiposity and restoration of insulin sensitivity (260). It is important to note that the leptin induced decreases in serum glucose, insulin and lipids at low dosages occur prior to changes in body mass (260), suggesting that leptin may have a direct effect on peripheral tissues such as skeletal muscle. Numerous peripheral tissues express leptin receptors (75, 331).

Leptin has been demonstrated to reduce TG content in various peripheral tissues such as liver, muscle and pancreatic cells (299) and to partition FA towards oxidation and away from storage in oxidative skeletal muscle (241, 242). Although the acute effects of leptin on glucose uptake and metabolism in muscle have been equivocal (64, 174, 274, 374), there is evidence of convergence between the leptin and insulin signaling pathways in this tissue (187, 242). Therefore, the direct effects of leptin on skeletal muscle metabolism appear well established. Skeletal muscle by virtue of its mass, is the major tissue responsible for insulin-stimulated glucose uptake and also accounts for a large proportion of whole-body energy metabolism, and may be an important tissue in

mediating leptin's effects on energy homeostasis.

In humans and rodents, serum concentrations of leptin are primarily dependent on adipose cell size (85). High levels of circulating leptin, even when normalized for body fat, characterize most cases of human obesity (85). This suggests the development of central and/or peripheral resistance to leptin in obesity. High-fat diets in rodents result in a diminished metabolic response to peripheral leptin injections (344), as well as impaired leptin transport across the blood brain barrier (19). In addition, several studies have demonstrated that high-fat diets lead to an increase in circulating leptin (9, 123, 204), which can occur in as little as 2 days (204). However, changes in plasma insulin subsequent to the consumption of high-fat diets may alter leptin production from adipocytes (86, 192). Thus, increases in plasma leptin may not be an accurate reflection of the development of leptin resistance. It is important to note that direct evidence for the development of leptin resistance during obesity in skeletal muscle does not exist.

Therefore, in this study we have utilized the pulse-chase technique in an isolated muscle preparation to examine the effect of high-fat diets on lipid metabolism, and specifically, whether diets high in n-6 and n-3 FAs alter the sensitivity of skeletal muscle to leptin. For the purpose of this study, we will define the development of leptin resistance as either an inability, or reduced ability of leptin to alter lipid metabolism in skeletal muscle (i.e. stimulate FA oxidation and decrease TG esterification). Diets high in saturated and n-6 polyunsaturated FAs (PUFA) induce insulin resistance in skeletal muscle (126, 375).

while partial substitution with up to 12% of the total kcal with n-3 FAs restores insulin sensitivity (323). The effect of high-fat diets of varying FA composition on leptin sensitivity in muscle has not been investigated. Therefore, we chose to utilize a dietary model that has been successfully employed to investigate the development of insulin resistance in muscle.

The isolated muscle preparation utilized in this study permits precise control of hormone and substrate concentrations, allowing us to study the effects of leptin in the absence of changes in the hormonal milieu as a result of the high-fat diets. We hypothesize that (1) diets high in PUFA will cause a compensatory increase in muscle lipid oxidation, (2) diets high in n-6 PUFA will reduce the sensitivity of oxidative muscle to the stimulatory effects of leptin on lipid metabolism, and (3) that the partial substitution of n-3 PUFA in the high-fat diet will restore the sensitivity of muscle to leptin.

Methods

Animals and Diets. Female Sprague-Dawley rats (Charles River Laboratories, Quebec, Canada) were used in all experiments. Upon arrival animals were weighed (165 ± 4 g) and assigned to individual cages in a controlled environment with a reverse 12-hour light/dark cycle. Animals were fed Purina rat chow ad libitum for a 7-day acclimation period. Animals were then assigned to one of 3 isocaloric and isonitrogenous diets for 4 weeks: high-safflower oil (HF-Saff; 60% kcal fat), high-safflower oil with fish oil (HF-Fish; n-6 48% kcal fat / n-3: 12% kcal fat), and a high-carbohydrate (CHO) control diet (CONT, 12% kcal fat, 16% kcal protein and 72% kcal CHO). The composition of these diets is summarized in Table 6. All diets met the American Institute of Nutrition guidelines for vitamin and mineral content. Diets were prepared as previously described (275), vacuum-sealed and stored at -20°C .

Table 6. Composition of experimental diets.

Ingredient	Diet		
	HCHO	HF-Saff	HF-Fish
	<i>g/kg diet</i>		
Casein (vitamin free)	150	200	200
Cornstarch	591	202	202
Sucrose	100	100	100
Safflower Oil	57.5	339	237
MaxEPA Oil	-	-	102
Fibre	50	90	90
Mineral Mix (AIN-93M)	35	48	48
Vitamin Mix (AIN-93VX)	10	14	14
Methionine	4.5	5.0	5.0
Choline bitartrate	2.0	2.0	2.0
Ethoxyquin	0.008	0.068	0.068
	<i>% kcal</i>		
CHO	72.1	23.8	23.8
Protein	16.1	16.1	16.1
Fat	11.8	60.0	60.0
<i>Caloric density (kcal/g)</i>	3.8	5.2	5.2

¹Casein, vitamin and mineral mixes, methionine, choline bitartrate and ethoxyquin were from ICN Biomedicals (Ohio). MaxEPA oil (R.P. Scherer Inc., Florida, USA) contained 30% *n*-3 fatty acids. Fibre was Solka Floc 100 (Fibre Sales and Development Corporation, Ohio). Safflower oil, granulated sucrose and cornstarch were purchased from Zehr's food markets (Guelph, Ont. Canada). Dashes denote zero amount.

Pulse-Chase Studies

Rats were anaesthetised with an intraperitoneal injection of sodium pentobarbital (6mg/100g body wt) and the SOL muscle was carefully dissected into longitudinal strips from tendon to tendon using a 27-gauge needle. Two SOL strips were utilized from each SOL muscle and placed in a 20 ml glass scintillation vial containing 3 ml of warmed (30°C), pregassed (95% O₂ / 5% CO₂, pH=7.4) modified Kreb's Henseleit buffer containing 4% FA free BSA (ICN Biomedicals), 2 mM pyruvate, and 0.5 mM palmitate. The pulse chase procedures utilized in this experiment were as previously described in this thesis (Chapter 3) with two exceptions. Unlike in Chapter 3 where there was no leptin present in the chase buffer incubation medium, in this set of experiments we were testing the effects of acute leptin treatment. Therefore, remaining muscle strips (following the wash) were transferred to the experimental (chase) phase of the incubation and exposed to either 0 or 10 µg/ml of murine leptin (Amgen, Thousand Oaks, CA, USA). This concentration was selected as it has previously been demonstrated to elicit a maximal response on skeletal muscle lipid metabolism (241). In a separate set of experiments it was confirmed that leptin did not degrade over the course of the incubations (0 min, 10.1 ± 0.9 µg/ml; 90 min, 9.3 ± 0.6 µg/ml, *n*=6). A second difference between the previous experiments was that the chase period was 90 min rather than 45 min.

Serum Leptin and Insulin. Blood samples were collected from animals at three time points (0, 2 and 4 wk). All samples were collected between 0900 and 1200 h in the fasted state to eliminate variability due to diurnal rhythm. Blood was collected from the tail vein after immersion in warm water for both the 0 and 2 wk time points. The final blood sample at 4 wk was collected via cardiac puncture following excision of the SOL. Serum leptin and insulin levels were assayed in duplicate using RIA kits specific for rat leptin and insulin (Linco Inc., St. Charles, MO).

Carcass Analysis. Frozen animal carcasses were cut into 4 or 5 small pieces and freeze dried for 5 days. Samples were finely ground and vacuum dried in an oven overnight. Percent body fat was assessed using the technique of Bligh and Dyer (37). Briefly, ~2 g of ground sample was added to a homogenizing tube containing 14 ml of distilled H₂O, 40 ml of methanol and 20 ml of chloroform and homogenized for 5 min. The homogenate was filtered, and the tube was rinsed with 20 ml of chloroform and 20 ml 1:1 of chloroform/methanol (vol/vol). The filtrate was added to a 100 ml graduated cylinder containing 25 ml of distilled H₂O. The filter flask was rinsed with 5 ml of 1:1 chloroform/methanol to remove any remaining lipids and the solution allowed to settle overnight. The top aqueous layer was aspirated, leaving the chloroform layer which was poured into a weighed 50 ml beaker. The chloroform layer was evaporated off in the fume hood leaving the lipid residue, which was weighed. Percent body fat for each animal was measured in triplicate.

Calculations and Statistics. The quantity of palmitate (nmol) esterified and oxidized was calculated from the specific activity of the incubation medium (i.e. radiolabeled palmitate in dpm / total palmitate in nmol). Intramuscular hydrolysis was calculated as the loss of preloaded ³H-palmitate from each lipid pool between paired soleus strips.

Results were analyzed using analysis of variance (ANOVA) procedures, and a Tukeys post hoc test was used to test significant differences revealed by the ANOVA. Significance was accepted at $p \leq 0.05$. All data are reported as mean $\pm$ SEM.

Results

Animal Characteristics:

Body composition. Body mass of rats fed CONT, HF-Fish and HF-Saff diets are shown in Fig.14A. Body mass did not differ among the groups at any time during the feeding protocol. Despite similar body masses, the mean percent body fat after 4 wk was significantly higher in animals fed the HF-Saff diet compared to animals on the other two diets (Fig. 14B).

Serum leptin and insulin. Serum leptin increased in all dietary groups over the 4 wk feeding period in relation to body mass gain ($r^2 = 0.35$, $p < 0.001$). Leptin levels were significantly elevated in the HF-Saff group at both 2 and 4 wk compared to the CONT (2 wk, $p < 0.001$; 4 wk, $p < 0.007$) and HF-Fish (2 wk, $p = 0.036$; 4 wk, $p = 0.037$) dietary groups (Fig. 15A). Serum leptin levels after 4 wk (normalized per gram of body fat) were significantly greater in the HF-Saff compared to CONT group ($p = 0.043$; Fig. 15B). Serum insulin increased significantly ($p < 0.001$) in both high-fat diet groups after 4 wk (HF-Saff, 2.5 ± 0.2 ng/ml; HF-Fish, 2.0 ± 0.15 ng/ml) while insulin levels in the CONT group remained unchanged (0.65 ± 0.12 ng/ml).

Basal fatty acid metabolism in response to a HF-Saff diet. Under basal conditions (i.e. absence of leptin), exogenous palmitate oxidation increased in the HF-Saff group by 45% ($p = 0.034$) compared to the CONT group (Fig. 16A). High-fat diets had no affect on basal palmitate esterification into any of the endogenous lipids, or hydrolysis. Total

incorporation of palmitate into SOL (lipid esterification plus oxidation) was unchanged following high-fat diets.

Metabolic Responses to Leptin:

Exogenous Palmitate Oxidation and Esterification. Exogenous palmitate oxidation in the CONT group was increased by 80% ($p = 0.0188$) in the presence of leptin (Fig. 16A). This stimulatory effect was eliminated in the HF-Saff and HF-Fish groups (Fig. 16A). Palmitate esterification into TG was reduced in the presence of leptin by 25% ($p = 0.043$) in the CONT and 26% ($p = 0.043$) in the HF-Fish group (Fig. 16B). Leptin did not decrease TG esterification in the HF-Saff group. Esterification of ^{14}C -palmitate to DG (Table 9) and PL (data not shown) was unaffected by leptin.

Intramuscular Lipid Hydrolysis and Oxidation. Hydrolysis of the DG (Table 7) and TG (Fig 17) pools was increased by 48% ($p = 0.04$) and 33% ($p = 0.043$), respectively, in the presence of leptin in the CONT group. Leptin had no effect on DG or TG hydrolysis in either the HF-Saff or HF-Fish groups. Leptin also had no affect on PL hydrolysis in any of the groups (data not shown). Endogenous lipid oxidation, monitored by the production of $^3\text{H}_2\text{O}$, was elevated in the presence of leptin in all groups but this increase did not reach statistical significance (Table 8).

Table 7. Degradation of endogenous ^3H -palmitate in DG and PL pools between start and end of 90 min chase in resting SOL muscle under basal conditions and with leptin

Diet	DG, nmol/g		PL nmol/g	
	Basal	Leptin	Basal	Leptin
HCHO	-4.8 ± 1.7	$-9.2 \pm 0.9^*$	3.3 ± 1.8	3.0 ± 1.0
HF-Saff	-2.9 ± 2.2	-4.3 ± 1.9	7.4 ± 2.4	5.9 ± 1.9
HF-Fish	-4.0 ± 2.0	-4.7 ± 1.5	4.7 ± 2.4	6.5 ± 1.7

Values are means $\pm$ SE; $n=12$. Negative values indicate a decrease of ^3H in pool.

*significantly different from basal conditions ($P=0.04$)

Table 8. Endogenous palmitate oxidation as monitored by the production of $^3\text{H}_2\text{O}$ over 90 min chase in resting SOL muscle under basal conditions and with leptin

Diet	$^3\text{H}_2\text{O}$ palmitate, nmol/g	
	Without Leptin	Leptin
HCHO	11.3 ± 2.4	14.1 ± 3.1
HF-Saff	12.4 ± 2.4	14.5 ± 2.6
HF-Fish	10.1 ± 2.1	15.5 ± 2.5

Values are means $\pm$ SE; $n=12$.

Table 9 Esterification of exogenous ^{14}C -palmitate to DG, and PL during 90 min chase in resting SOL muscle under basal conditions and with leptin

Diet	DG, nmol/g		PL nmol/g	
	Without Leptin	Leptin	Without Leptin	Leptin
HCHO	11.5 ± 1.1	11.4 ± 1.4	20.3 ± 2.0	19.2 ± 1.4
HF-Saff	13.9 ± 2.4	15.5 ± 3.9	24.1 ± 2.5	23.8 ± 2.7
HF-Fish	15.8 ± 3.4	17.2 ± 5.8	22.4 ± 2.3	25.5 ± 2.6

Values are means $\pm$ SE; $n = 12$.

Figure 14. Body mass of animals over 4 wk feeding protocol (A) and percent body fat (B) following 4 wk of diets. Values are means $\pm$ SE, $n=12$. a, significantly different from CONT or HF-Fish diet.

Figure 15. Serum leptin concentrations over 4 wk feeding protocol (A) and serum leptin concentrations normalized per gram body fat (B). Values are means $\pm$ SE, $n=12$. a, Significantly different from CONT and HF-Fish diet; b, Significantly different from CONT.

Figure 16. (A) Exogenous palmitate oxidation and (B) palmitate esterification to TG during 90 min in the presence or absence of leptin. Values are means $\pm$ SE, $n=12$. a, Significantly different from basal condition without leptin; b, Significantly different from CONT diet.

Figure 17. Triacylglycerol hydrolysis during 90 min in the presence or absence of leptin. Values are means $\pm$ SE, $n=12$. a, Significantly different from basal condition without leptin.

Figure 18. Fatty acid partitioning ratio of incorporated palmitate. Values are means $\pm$ SE, $n=12$. a, Significantly different from basal condition without leptin, b, Significantly different from CONT diet.

Figure 19. (A) Triacylglycerol turnover (synthesis - hydrolysis) and (B) total palmitate oxidation (endogenous + exogenous oxidation) during 90 min in the presence or absence of leptin. Values are means $\pm$ SE, $n=12$. a, significantly different from basal condition without leptin.

Discussion

In this study, we utilized the pulse-chase technique in isolated SOL strips to examine the effect of 4 wks of high-fat diets on muscle lipid turnover and oxidation, and more specifically, whether the consumption of high-fat diets resulted in the loss of leptin's effects on muscle lipid metabolism. The use of an isolated muscle preparation allows for the examination of the direct consequences of a high-fat diet on the responsiveness of muscle to leptin in the absence of other metabolic perturbations (i.e. altered circulating insulin, FA, etc.). To our knowledge, this is the first study to demonstrate direct evidence of the development of leptin resistance in peripheral tissues such as skeletal muscle. Several novel observations were made in the present study: 1) diets high in safflower oil (n-6 PUFA) induce a compensatory increase in basal lipid oxidation in oxidative muscle; 2) this compensation is not observed when 12% of the safflower component is replaced with fish oil (n-3 PUFA); 3) in CONT animals, leptin significantly stimulates the hydrolysis of muscle DG and TG, and repartitions FA towards oxidation and away from esterification; 4) the stimulatory effects of leptin on muscle lipid oxidation and hydrolysis were eliminated following consumption of both high-fat diets; 5) the inhibitory effect of leptin on lipid esterification was eliminated in the HF-Saff group, but was restored when fish oil was included in the high-fat diet; and 6) the increase in serum leptin during the 4 wk dietary treatment was greater than CONT only in the HF-Saff group, suggesting that increases in circulating leptin are not an accurate indication of leptin resistance in skeletal muscle.

Effect of High-fat Diets on Muscle Lipid Metabolism. An increase in basal whole-body lipid oxidation in response to high-fat diets has been demonstrated in rodents (67) and humans (294, 295). However, there is relatively little evidence documenting the effect of FA composition on muscle lipid oxidation. In this study, we provide evidence that 4 wks of feeding a HF-Saff, but not a HF-Fish diet, leads to a compensatory increase in exogenous palmitate oxidation. This discrepancy is unexpected given existing evidence that diets high in n-3 PUFA stimulate whole-body lipid oxidation in humans (90) and stimulate muscle CPT I activity in rats (269). However, we are unaware of any studies, which have directly examined the effects of PUFA composition on muscle lipid metabolism. The increase in lipid oxidation in the HF-Saff animals also seems paradoxical given the increase in body fat in this group. This may in part be due to the fact that our observations of increased lipid oxidation were made in oxidative muscle, and may not be relevant to the whole organism. Alternatively, it is possible that a greater degree of insulin resistance was induced in skeletal muscle in the HF-Saff group, which may have provided more glucose for disposal in adipocytes. A recent study has clearly demonstrated that high fat diets induce insulin resistance in skeletal muscle, but not adipose tissue (361).

While there is a paucity of information regarding the effects of dietary fat on the regulation of muscle lipid metabolism, the beneficial effects of fish oil on basal and insulin-stimulated glucose utilization are well documented. Therefore, under our

experimental conditions, it is possible that glucose utilization was most severely impaired in the High-Saff group, which may have resulted in a greater reliance on lipid oxidation. This is supported by a recent study in humans, which found that safflower oil impaired pyruvate dehydrogenase activity, but fish oil did not (173).

In addition, it may be possible that the HF-Saff diet increased muscle lipid oxidation through an increase in the expression of uncoupling protein 3 (UCP-3), which has been shown to be increased by FA and leptin (135, 357). In the present study, serum leptin was elevated to the greatest extent in the High-Saff group raising the possibility that UCP-3 expression was increased, thus enhancing lipid oxidation. Alternatively, it may be possible that different PUFA have variable direct effects on the expression of UCP-3. However, studies examining the effect of high-fat diets on UCP-3 expression in rodent skeletal muscle have been equivocal (87, 219). Because the expression of uncoupling proteins was not measured in the present study, our hypothesis cannot be confirmed. We are also unaware of any studies which have examined the effects of PUFA composition on uncoupling protein expression in skeletal muscle. Our study suggests that the composition of the high-fat diet may have an important effect on uncoupling protein expression, and warrants further investigation.

Effect of Leptin on Lipid Metabolism in Skeletal Muscle. In this study we demonstrate that leptin increases exogenous lipid oxidation by 80% while reducing lipid esterification by 25% in CONT animals. Thus, the ratio of FA esterification to oxidation was reduced

by 140% ($p < 0.001$), indicating a repartitioning away from esterification and towards oxidation (Fig 18). These findings are similar to data recently reported by Muoio et al. (241, 242) in mouse soleus muscle. In an extension to the findings of Muoio et al. (241, 242), we have also provided the first direct evidence that leptin stimulates the hydrolysis of the intramuscular lipids, DG and TG. Furthermore, our results demonstrate that leptin's effects on lipid metabolism are not specific to the FA oleate as used previously (241, 242).

To date, the mechanism by which leptin acutely repartitions FA towards oxidation in skeletal muscle is unknown. In pancreatic cells incubated for 2 d in leptin, a 20% decrease in the expression of ACC2 was observed which coincided with an increase in lipid oxidation (373). This is presumably due to a decrease in malonyl CoA, which is a potent inhibitor of CPT I (233); however, direct changes in malonyl CoA were not measured. Although the role of malonyl CoA in regulating muscle lipid oxidation remains controversial, particularly in human muscle (248), recent evidence using isolated rodent muscle has indicated that acutely decreasing the activity of ACC2 directly leads to an elevation in lipid oxidation (167). Leptin was also observed to significantly increase the expression of acyl-CoA oxidase and CPT I (373). There is no data regarding the potential effects of leptin on rate-limiting enzymes regulating TG hydrolysis (e.g. hormone sensitive lipase); however, a decrease in GPAT expression in pancreatic islet cells has been observed (373). In the present study, total incorporation of FA (esterification and oxidation) was unaffected by leptin in CONT muscle (no leptin, 220.4

± 32.0 ; leptin, 184.5 ± 16.9 nmol/g), suggesting that the transport of FA across the sarcolemma is not affected. Interestingly, the effect of leptin on TG turnover (synthesis - degradation) was greater than the increase in total (endogenous and exogenous) palmitate oxidation (Fig 19), indirectly suggesting that leptin may have a direct effect on the synthesis and hydrolysis of intramuscular TG.

Development of Leptin Resistance:

Effect of High-Fat Diets on Serum Leptin. It has been suggested that the elevation in circulating leptin levels in obesity and subsequent to the consumption of high-fat diets may reflect the development of resistance to leptin's metabolic effects. Although leptin resistance at the level of the blood brain barrier has been demonstrated in response to high-fat diets (19, 344). High-fat diets may result in elevated serum leptin due to a spontaneous increase in energy consumption (192) or secondary to increases in insulin (67) which has been demonstrated to stimulate leptin production in adipocytes from rodents and humans (126, 192). Furthermore, the effects of high-fat diets on circulating leptin levels are variable (9, 65, 123, 165, 204, 327), which may be due to the type of fat used in the diet. Supplementation with docosahexanoic acid in rats has been demonstrated to reduce adipocyte leptin mRNA (272). This may explain the findings of the present study, that a HF-Saff diet leads to a significant elevation in serum leptin at both 2 and 4 wks compared to CONT and HF-Fish oil groups.

Direct Evidence for Leptin Resistance in Skeletal Muscle. One of the major objectives of the present study was to determine whether high-fat diets result in an impaired response of skeletal muscle to leptin. This phenomenon has not been investigated in any study to date. In the present study, we have defined leptin resistance in skeletal muscle as an impaired ability of leptin to stimulate FA oxidation and TG hydrolysis, and to blunt TG esterification.

Leptin has been demonstrated to have profound effects on lipid metabolism in skeletal muscle (241, 242). However, the present study indicates that these effects can be almost completely eliminated following 4 wks of diets high in n-6 and n-3 PUFA. It must be acknowledged that the basal increase in FA oxidation in the HF-Saff group complicates the interpretation of the lack of further stimulation in the presence of leptin. However, we have previously demonstrated that resting soleus muscle can oxidize palmitate at a rate of ~100 nmol/g/hr when the concentration of palmitate is increased to 1.0 mM (104). The rate of ~20 nmol/g in 90 min observed in the HF-Saff group is well below the capacity of soleus to oxidize FA at rest. Therefore, we interpret the inability of leptin to further stimulate palmitate oxidation in soleus from the HF-Saff group as a resistance to the effects of leptin. It is important to note that although substitution of fish oil did not increase fat oxidation it did restore leptin's inhibitory effect on TG synthesis. Thus, the inclusion of dietary n-3 PUFA may help counter some of the deleterious effects of n-6 PUFA on leptin sensitivity in muscle. Based on the results of the present study, the possibility that a higher substitution of fish oil would have elicited a greater restoration

on the sensitivity of muscle to leptin cannot be eliminated.

Numerous studies have demonstrated that high-fat feeding results in a reduction in insulin sensitivity in skeletal muscle (126, 375). It is important to note that these results are consistent even in studies such as our own in which animals are pair-fed to minimize differences in body mass (323, 361). Insulin resistance as a result of high-fat feeding has been correlated with an increase in intramuscular TG (106, 266). Partial substitution of n-6 PUFA with n-3 PUFA has been found to reduce intramuscular TG accumulation and restore insulin sensitivity (322, 323). The restoration of insulin sensitivity in *ob/ob* mice following leptin administration also appears to be related to a reduction in intramuscular TG (57). Changes in membrane fluidity as a result of a relative reduction in the percentage of highly unsaturated n-3 PUFA in phospholipids has also been associated with impaired activation of the insulin receptor (126, 184, 186, 375). It is reasonable to hypothesize that the leptin receptor would be similarly affected, although direct evidence for altered leptin signaling in response to changes in membrane lipid composition are lacking.

Summary. In conclusion, the present study has demonstrated that diets high in n-6 PUFA stimulate basal lipid oxidation in skeletal muscle, but not when fish oil is substituted for 12% of the total kcal. Serum leptin was significantly elevated by the diet high in safflower oil, indirectly suggesting the development of leptin resistance in this group. However, pulse-chase studies using incubated SOL strips revealed that the anti-lipogenic

effects of leptin were virtually eliminated in both high-fat diets. Thus, we hypothesize that during the development of obesity, skeletal muscle becomes resistant to the effects of leptin resulting in the accumulation of intramuscular TG. This may be an important initiating step in the development of insulin resistance common in obesity.

Chapter 6: Leptin Increases Fatty Acid Oxidation in Lean but not Obese Human

Skeletal Muscle: Evidence of Peripheral Leptin Resistance

Introduction

Skeletal muscle is the major tissue contributing to basal metabolic rate and is also the primary tissue responsible for whole body glucose and FA metabolism (377). Skeletal muscle from the obese has an increased content of TG (139, 177) which is strongly correlated to reduced insulin sensitivity (177, 256). The mechanisms by which obese individuals increase TG storage in skeletal muscle are currently unknown, but may be due to several factors, including 1) increased FA uptake 2) a repartitioning of incorporated FA towards esterification and away from oxidation, and 3) a reduced rate of TG hydrolysis.

Fatty acid metabolism in resting rodent skeletal muscle is regulated by several hormones, including leptin (241, 316), insulin (105) and epinephrine (264). Leptin, a circulating 16-kDa adipocyte-derived protein, regulates food intake and energy expenditure in animal models (144). It has also been proposed that a primary role of leptin may be to prevent the accumulation of lipids in peripheral tissues such as skeletal muscle (341). In agreement with this proposal, we and others have demonstrated both acutely (241, 316) and chronically (315) that in rodent skeletal muscle leptin reduces intramuscular TG by increasing FA oxidation and TG hydrolysis, while reducing FA esterification. The effects of leptin on human skeletal muscle metabolism, however, are unknown.

Human obesity is characterized by high levels of circulating leptin (207), which suggests the development of central and/or peripheral leptin resistance. We have recently demonstrated that in rodents, 4 wks of high fat feeding can induce leptin resistance in skeletal muscle as demonstrated by the elimination of leptin's stimulatory effect on FA metabolism (316). Thus we have hypothesized that during the development of human obesity, skeletal muscle becomes resistant to leptin, leading to the accumulation of intramuscular TG and the development of insulin resistance. However, direct evidence of leptin resistance in human skeletal muscle does not exist.

Therefore, the principal purpose of this study was to examine the acute effects of leptin on human skeletal muscle FA metabolism. We hypothesized that leptin would stimulate FA oxidation and TG hydrolysis while suppressing FA esterification into TG in lean human skeletal muscle, but that these effects would be blunted in the obese. To monitor FA metabolism, we utilized an isolated rectus abdominus muscle preparation previously described by Dohm et al. (98) combined with the dual-label pulse chase technique which has been used to examine FA metabolism in rodent skeletal muscle (104, 264, 316). Unlike a previous study that assessed FA oxidation rates in obese human skeletal muscle homogenates (185), the present study utilizes intact muscle strips. While other studies have examined rates of skeletal muscle FA metabolism in obese humans *in vivo* (80, 176), metabolic complications associated with the obese state and alterations in the hormonal milieu makes it difficult to assess the contribution of individual hormonal factors. The isolated muscle preparation used in this study permits precise control of

hormone and substrate concentrations, allowing us to study the effects of obesity and leptin in the absence of other metabolic perturbations.

Methods

Human Subjects. The participants were 8 lean ($\text{BMI} \leq 27 \text{ kg/m}^2$, $24.8 \pm 0.5 \text{ kg/m}^2$) and 9 obese ($\text{BMI} \geq 30.0 \text{ kg/m}^2$, $33.0 \pm 1.5 \text{ kg/m}^2$) nondiabetic women (Table 10). Subjects were admitted to McMaster Health Sciences Center for a variety of abdominal surgical interventions and gave informed written consent before participating. Experimental protocols were approved by the McMaster University Ethics Committee. None of the subjects had any diseases or had taken any medications known to alter carbohydrate or lipid metabolism in the six months before surgery. All subjects had maintained a constant body mass during the year preceding surgery. Four women in the obese group and two women in the lean group were postmenopausal. Of these women, two obese subjects and one lean subject were receiving estrogen treatment. One woman from each group was consuming oral contraceptives. Muscle lipid metabolism did not differ in these subjects, and they were thus included in data analyses. After an overnight fast (12-18 hrs), general anesthesia was induced with a short-acting barbiturate, and maintained by a fentanyl and rocuronium volatile anesthetic mixture. Venous blood samples were then collected in 5 ml heparinized tubes during anesthesia. Muscle strip preparation was completed as previously described (98) with minor modifications. A biopsy of 3 x 2 x 1 cm from the rectus abdominus muscle was excised perpendicular to the direction of the muscle fibers, clipped at resting length and placed in oxygenated, ice-cold Krebs-Henseleit buffer for

transport to the laboratory (~5 min). Four muscle strips weighing ~25 mg were separated from the muscle sample, and clipped at resting length.

Muscle-Viability. In preliminary experiments, ATP, phosphocreatine (PCr) and total creatine (Cr) were measured spectrophotometrically (27) to ensure muscle viability. Muscle strips were rapidly frozen in liquid N₂ after excision (control), or after 30 or 90 min of incubation under the same conditions in which pulse-chase experiments were conducted.

Pulse-Chase Studies. All pulse-chase procedures including the temperature and incubation times and concentrations were as previously described in Chapter #5 with one minor exception; recombinant human leptin was used in place of recombinant murine leptin. The measurement of endogenous and exogenous palmitate as well as lipid extractions were completed as previously described in Chapter #3. Calculations and Statistics were completed as described in Chapter #5.

Results

Subjects. Subject characteristics are shown in Table 10. Obese individuals had significantly greater body mass (+34%, $P<0.001$) and body mass index (+35%, $P<0.001$) compared to lean subjects. Fasting levels of plasma glucose, FAs, β -hydroxybutyrate, and insulin were not significantly different between lean and obese women ($P>0.05$). Leptin levels were significantly elevated (+140%, $P=0.002$) in obese individuals and were positively correlated to BMI ($r=0.73$, $P=0.016$) as previously demonstrated (5).

Table 10. Clinical Characteristics and Fasting Plasma Values of Lean and Obese Women.

	Lean	Obese	P
Age (yr)	48.7 $\pm$ 4.0	55.1 $\pm$ 6.4	NS
Weight (kg)	66.4 $\pm$ 1.6	89.6 $\pm$ 3.2	<0.001
Body mass index (kg/m ²)	24.8 $\pm$ 0.5	33.0 $\pm$ 1.5	<0.001
Glucose (mM)	6.1 $\pm$ 0.3	6.0 $\pm$ 0.3	NS
Free fatty acids (mM)	1.00 $\pm$ 0.16	1.02 $\pm$ 0.23	NS
Glycerol (mM)	0.09 $\pm$ 0.02	0.15 $\pm$ 0.01	0.03
B-Hydroxy-butyrate (mM)	0.53 $\pm$ 0.12	0.38 $\pm$ 0.16	NS
Insulin (μ U/ml)	8.32 $\pm$ 1.12	9.38 $\pm$ 3.69	NS
Leptin (ng/ml)	8.20 $\pm$ 1.77	19.74 $\pm$ 1.6	0.002

$n = 6-8$

Values are mean $\pm$ SEM

Muscle Strip Preparation Viability. Muscle viability was preserved during incubations, based on the maintenance of ATP, PCr and total Cr contents at both 30 and 90 min time points (Table 11).

Table 11. ATP, PCr, Cr and total Cr levels in control (quickly frozen) and incubated muscle strips from nonobese subjects

	Control (n=5)	30 min (n=8)	90 min (n=5)
ATP	19.2 ± 1.3	17.6 ± 1.0	17.0 ± 1.3
PCr	51.8 ± 1.3	46.2 ± 1.3	47.4 ± 2.6
Cr	26.3 ± 0.8	27.6 ± 0.5	23.0 ± 3.1
Total Cr	78.1 ± 1.1	73.8 ± 1.3	70.4 ± 3.9

Values are mean ± SEM μ mol/g dry wt

Skeletal Muscle Fatty Acid Metabolism

Lean vs. Obese. In the absence of leptin, exogenous and endogenous palmitate oxidation were not different between lean and obese subjects (Fig. 20A and 20B). However, obese muscle had significantly higher levels of total FA uptake (+72%, $P=0.038$; Fig. 20C), primarily due to increased rates of FA esterification into TG (+102%, $P=0.042$; Fig. 20D). Fatty acid esterification into PL was significantly elevated (+96%, $P=0.031$) in the obese while esterification into DG was not different (data not shown). There was no change in the hydrolysis of TG, DG or PL between lean and obese groups (data not shown). Total FA uptake ($r=0.54$, $P=0.027$) and esterification to TG ($r=0.58$, $P=0.014$) were positively correlated to BMI (Fig. 21). It should be noted that due to the small number of subjects if the subject with a BMI of ~43 was removed from the regressions of

total FA uptake and TG esterification there was no longer a significant correlation ($P=0.186$ and $P=0.085$, respectively). The ratio of palmitate esterification (PL, DG, TG) to oxidation was significantly elevated in obese muscle (+49%, $P=0.05$), indicating a partitioning of incorporated FA towards storage (Fig. 22).

Metabolic responses to leptin in lean skeletal muscle. Exogenous and endogenous palmitate oxidation (Fig 20A and 20B) was significantly elevated (+150 and +103% respectively, $P<0.03$) in the presence of leptin. Leptin had no effect on FA uptake (Fig. 21A), TG esterification (Fig. 21B), or the hydrolysis of TG and DG pools (data not shown). Leptin treatment resulted in a significant reduction in the FA esterification to oxidation ratio (-50%, $P=0.016$; Fig. 22).

Metabolic responses to leptin in obese human skeletal muscle. FA oxidation was not increased by leptin in obese skeletal muscle strips (Fig. 20A and 20B). Leptin was also without effect on total FA uptake (Fig. 21A), TG esterification (Fig. 21B), or hydrolysis of TG and DG pools (data not shown), or the esterification to oxidation ratio (Fig 22).

Figure 20. Exogenous (A) and endogenous (B) palmitate oxidation in resting human muscle strips from lean and obese women. Values are means $\pm$ SE, $n=8$ lean and $n=9$ obese. ^a Significantly different from control.

Figure 21. Total palmitate uptake (A) and triacylglycerol esterification (B) in resting human muscle strips from lean and obese women. Values are means $\pm$ SE, $n=8$ lean and $n=9$ obese. ^b Significantly different from lean

A

B

Figure 22. Relationship between (A) total palmitate uptake, and (B) triacylglycerol esterification in muscle strips from lean and obese women. Values are means $\pm$ SE, $n=8$ lean and $n=9$ obese.

Figure 23. Fatty acid partitioning ratio of incorporated palmitate. Values are means $\pm$ SE, n=8 lean and n=9 obese. a Significantly different from control and b significantly different from lean

Discussion

In this study we used the pulse-chase technique in isolated human skeletal muscle from lean and obese women to examine the effect of obesity and leptin on FA metabolism. Our results demonstrate that, 1) in obese human skeletal muscle, there is a significantly greater rate of FA uptake and esterification compared to skeletal muscle of lean individuals, and 2) that leptin stimulates FA oxidation in skeletal muscle from lean, but not obese subjects, providing the first direct evidence of peripheral leptin resistance in obese humans. Thus, an increased rate of FA uptake and esterification, coupled with an inability for leptin to stimulate FA oxidation may lead to the increased intramuscular TG content observed in obesity. While one of the benefits of the isolated muscle preparation is the ability to precisely control hormonal and substrate concentrations and thus establish a direct effect of leptin, it should also be noted that the non-physiological conditions imposed in such a preparation (i.e. high leptin, absence of insulin and other hormonal factors) makes it difficult to extrapolate our findings to the *in vivo* condition.

Lean vs. Obese Differences in Basal Rates of FA Metabolism. In this study, there was a significantly greater rate of FA uptake and TG esterification in skeletal muscle of the obese. However, obesity did not result in altered basal rates of endogenous or exogenous FA oxidation. Taken together, this results in an elevated esterification to oxidation ratio in obese human skeletal muscle indicating a greater rate of FA storage, and is in agreement with previous findings demonstrating elevated levels of intramuscular TG with obesity (139, 177). Previous studies have demonstrated increased content of skeletal

muscle FA transporters in obese rats (FAT/CD36 and FABPpm) (210) and humans (FABPpm) (305), suggesting that the enhanced rate of FA esterification is due at least in part to increased FA transport.

Our finding of unaltered rates of FA oxidation differs from previous reports of reduced FA oxidation in the obese state (176, 185). We have previously demonstrated that insulin reduces FA oxidation by 38% in resting rodent skeletal muscle (105), and in humans, insulin reduces whole-body FA oxidation by 63% (23). Thus, reduced rates of FA oxidation previously reported in human obesity (176) may be due in part to insulin's suppression of FA oxidation. Reduced rates of FA oxidation have also been reported in muscle homogenates from obese humans in the absence of insulin (185). However, it is difficult to compare the findings of Kim et al. (185) to our own, due to the disruption of the sarcolemma, and the use of a different muscle type (vastus lateralis) in their study. Furthermore, it should be noted that the subjects used in our study (BMI, 33.0 kg/m²) were less obese than the subjects used by Kim et al. (BMI, 38.3 kg/m²) suggesting that the blunted rates of FA oxidation may not occur at lower levels of obesity. Thus, it is possible that during the development of obesity, the primary defect occurs at the level of the plasma membrane due to increased expression of the FA transporters, resulting in a disproportionate increase in FA transport, and hence esterification in the myocyte.

Effects of Leptin on Fatty Acid Metabolism in Human Skeletal Muscle. Several *in vitro* studies have examined the effects of recombinant leptin on peripheral tissues of rodents.

Leptin decreases TG stores in adipocytes (341), pancreatic islets (299) and skeletal muscle (299). A reduction in skeletal muscle TG by chronic leptin treatment is associated with increased insulin sensitivity (367). We have previously shown that leptin reduces skeletal muscle TG by reducing TG esterification and increasing FA oxidation and TG hydrolysis in oxidative rodent skeletal muscle (316). In this study we have demonstrated for the first time a direct effect of leptin on human skeletal muscle. Leptin significantly enhanced both endogenous and exogenous rates of FA oxidation, but failed to increase TG hydrolysis or suppress FA esterification, suggesting a possible species difference. Importantly, leptin did not stimulate FA oxidation in skeletal muscle of obese women, demonstrating the development of peripheral leptin resistance.

Human obesity is characterized by elevated serum leptin levels (85), leading to the hypothesis that obese persons are resistant to leptin. In rodents, high-fat diets increase serum leptin levels and reduce leptin sensitivity in skeletal muscle (316). In rodents, recombinant leptin administration is associated with dose-dependent reductions in food intake and body weight (139); however, the effect of recombinant leptin administration in obese humans has been less successful (144, 367). Our findings provide direct evidence of leptin resistance in human skeletal muscle. While the cause of leptin resistance in obese humans remains unknown, future studies may examine the expression of SOCS-3, a member of the suppressors of cytokine signaling family, which has been demonstrated to potently inhibit leptin signaling in rodents (34).

In summary, the present study has demonstrated that obese human skeletal muscle has significantly greater rates of FA uptake than muscle from lean subjects and that leptin fails to stimulate FA oxidation in the obese. This reduced capacity for leptin to stimulate FA oxidation coupled with an increased potential for FA uptake may lead to the accumulation of intramuscular TG commonly observed in obesity. While the mechanism by which skeletal muscle becomes resistant to leptin is unknown, our data indicates that resistance occurs at an early stage in the development of obesity, and may precede impairments in FA oxidation.

Chapter 7: General Summary

The discovery of leptin 7 years ago created a resurgence in the study of body mass regulation. Initially most investigation focused on the effects of leptin on central pathways and the regulation of food intake. However, the widespread distribution of the different leptin receptors suggest that leptin has wide ranging implications for the regulation of multiple systems in almost all areas of physiology. This thesis has examined a small segment of leptin's comprehensive role, but one that is nonetheless of biological importance. As previously discussed, skeletal muscle plays an important role in regulating glucose and FA metabolism and the relationship between fat/carbohydrate interaction also has important implications for the development of insulin resistance and type 2 diabetes. Therefore, the regulation of skeletal muscle FA metabolism by leptin could be considered critical in mediating leptin's effects on obesity and insulin sensitivity.

The purpose of the initial study of this thesis (Chapter 3) was to examine the effects of chronic leptin treatment on rodent skeletal muscle FA metabolism as chronic leptin treatment had previously been demonstrated to reduce TG stores in several tissues. To study the effects of moderate hyperleptinemia we removed soleus muscles following 14 days of treatment and then measured FA metabolism at rest and during contraction using pulse-chase procedures. It's important to note that in this study, leptin was not present in the incubation medium during the pulse-chase experiments. Therefore, alterations in FA metabolism were the result of the prior chronic exposure to elevated leptin levels *in vivo*.

In this experiment we demonstrated that reduced intramuscular TG following leptin treatment was due to the stimulation of FA oxidation and TG hydrolysis. These effects on FA oxidation and TG hydrolysis were not attributed to an increased oxidative capacity as assessed by the measurement of the mitochondrial enzymes CS and β -HAD nor increased protein expression of HSL respectively. This led to the development of an alternative hypothesis which suggested that leptin may be upregulating FA oxidation by increasing the expression of AMPK leading to reduced ACC2 activity and therefore lower malonyl-CoA levels resulting in an increased flux through CPT-1. An upregulation of AMPK also increases UCP-3 protein expression, which may also increase FA oxidation. We now have direct evidence supporting the hypothesis that chronic leptin treatment increases AMPK expression although we were unable to detect changes in malonyl-CoA levels (Steinberg et al. unpublished observations). In regards to TG hydrolysis, several studies have demonstrated that HSL activity is regulated covalently by phosphorylation. Therefore the unaltered protein expression may not reflect the activity of the enzyme. Of note is evidence suggesting that AMPK activation reduces HSL activity in adipose tissue (129, 326), which in skeletal muscle seems counter intuitive based on AMPK's role of stimulating ATP production. Future studies will need to investigate the role of AMPK in the regulation of HSL in skeletal muscle and to measure the activity of HSL following chronic leptin treatment.

In Study 2, the mechanisms by which chronic leptin treatment reduces intramuscular TG were measured, including FA transport in red and white skeletal muscles. While in Study

1 there was a trend towards reduced FA uptake in resting soleus muscle following leptin treatment this is not an exclusive measure of FA transport. Therefore by preparing giant sarcolemmal vesicles from red and white gastrocnemius following chronic leptin treatment we were able to directly measure reduced rates of FA transport independent of metabolism. This reduction in FA transport was mediated by the downregulation of both FAT/CD36 and FABPpm at the level of the plasma membrane. Interestingly, the downregulation of both proteins in the membrane was mediated in different ways. That is, while leptin reduced the mRNA content and total protein expression of FAT/CD36, leptin did not alter FABPpm mRNA or total protein expression. These findings suggest that FABPpm, like FAT/CD36, is capable of translocation and that these proteins are regulated by leptin independently although the end result (reduced transporter expression in the membrane) is the same. These studies highlight the multiple sites of leptin action and the potent lipopenic role of leptin which reduces intramuscular TG by: 1) limiting the entry of FA into the myocyte through downregulation of the transport proteins, 2) upregulating FA oxidation despite a reduced supply, and 3) promoting the hydrolysis of existing intramuscular TG stores.

A significant limitation of the *in vivo* studies discussed above is that artificially elevating leptin levels leads to a reduction in circulating insulin. This is problematic because of the opposing effects that leptin and insulin have on skeletal muscle FA metabolism. For example while leptin increases FA oxidation and TG hydrolysis, insulin suppresses both of these activities while also stimulating TG esterification and FA transport. Therefore, it

is impossible to state confidently that, alterations in FA metabolism and transport following leptin treatment were due exclusively to elevated leptin levels and not due to a reduction in insulin. Follow-up studies, addressing this issue by maintaining insulin at physiological concentrations during leptin treatment are currently underway in our laboratory.

Despite the pronounced effects of leptin treatment in animal models of obesity, elevated levels of leptin do not have similar effects in obese humans. In obese humans, elevated levels of circulating leptin indicate a state of central and/or peripheral leptin resistance but direct evidence of leptin resistance in skeletal muscle did not exist before Study 3 of this thesis (Chapter 4). In this study direct evidence of skeletal muscle leptin resistance was demonstrated in animals fed a high-fat diet as assessed by the blunting of leptin's acute stimulatory effects on skeletal muscle FA metabolism. Of interest was the finding that substitution with n-3 FA did not fully restore leptin sensitivity (only leptin's effect on TG esterification) as hypothesized from insulin studies. It might be interesting to repeat this study with a higher dose of fish-oil to determine whether all of leptin's effects could be restored. From insulin studies, it is also known that reduced insulin sensitivity caused by high-fat feeding can be reversed if animals are endurance trained during the feeding protocol (180, 183). Therefore, an interesting follow up to our findings of leptin resistance following high-fat feeding would be to examine whether the development of leptin resistance could be reversed with interventions known to alter insulin sensitivity such as endurance training.

Despite the immense number of publications presented on leptin in rodents there have been few studies completed in humans. Most of these studies in humans have been reports of associations between leptin levels and various physiological and disease states and only a few have been designed to directly evaluate the role of leptin in humans. Therefore, in Study 4 (Chapter 6) we studied the effects of acute leptin exposure on human skeletal muscle FA metabolism. A secondary purpose of this study was to determine whether skeletal muscle of obese humans was leptin resistant as we hypothesized from our previous findings in rodent skeletal muscle. Under basal conditions we found that obese human skeletal muscle has much higher rates of FA uptake while having normal rates of FA oxidation when compared to lean subjects. This suggests that increased FA transport and the expression of FA transport proteins (Bonen et al. unpublished findings) is the principle defect which contributes to the accumulation of intramuscular TG common in obesity. This study provided the first evidence of a direct effect of leptin on stimulating FA oxidation in lean human skeletal muscle. In addition, it also established direct evidence of leptin resistance in skeletal muscle of obese humans as demonstrated by the inability of leptin to stimulate FA oxidation. Because of the isolated nature of this study it is difficult to extrapolate my findings to lean and obese humans *in vivo* because of the interaction between leptin and other circulating hormones. Therefore future studies should examine the acute effects of leptin treatment on skeletal muscle FA metabolism in lean and obese humans *in vivo* using stable isotopic tracers.

Future studies should also investigate the mechanisms by which leptin stimulates FA oxidation as well as the molecular mechanisms for leptin resistance in the obese. i.e. SOCS-3 a protein that has been implicated in the development of leptin resistance. Leptin signaling through AMPK and its effect on downstream targets such as ACC, GPAT and HSL should be investigated in both lean and obese humans. If leptin is found to activate this pathway in lean but not obese humans due to leptin resistance at some point between the leptin receptor and AMPK then novel peptides capable of stimulating AMPK directly in the obese that are also safe for human consumption could be developed. Therefore, it might be predicted that these novel peptides would be capable of stimulating FA metabolism in skeletal muscle and therefore help promote weight loss and improve skeletal muscle insulin sensitivity in the obese.

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Appendices

Appendix 1. FA transport and the Giant Sarcolemmal Vesicle Preparation

The giant sarcolemmal vesicle preparation used in this thesis allows for a true measurement of FA transport, independent of metabolism. It is critical to divorce metabolism from transport in order to get a true measure of FA uptake so that saturation kinetics of FA uptake are not due to limitations related to intracellular FA metabolism. Unlike other preparations that have been used previously to measure FA uptake, such as hepatocytes, cardiomyocytes, and adipocytes, giant sarcolemmal vesicles provide many advantages to present a true measure of FA transport rates. We have shown that in giant sarcolemmal vesicles, (a) initial rates of FA uptake can be determined due in part to the elimination of backflux because of the larger volume (diameter 10- 15 μm) of giant sarcolemmal vesicles (See Figure 23) compared with small sarcolemmal vesicles ($<1 \mu\text{m}$), (b) giant vesicles contain FABPc in excess, which provides for a large intravesicular FA sink, (c) all of the palmitate taken up by the vesicles is fully recovered as unesterified palmitate (i.e. none of the palmitate taken up is esterified, oxidized or associated with the plasma membrane), and (d) vesicles are 100% oriented right side out oriented. Therefore, the use of the giant sarcolemmal vesicle preparation used in this thesis provides an appropriate model with which to examine leptin's effects on FA transport in skeletal muscle. A potential disadvantage of this technique is the unphysiological nature of the giant sarcolemmal vesicles, as they do not contain T-tubules normally found within skeletal muscle.

Figure 24. Photograph of giant sarcolemmal vesicles as viewed under a phase contrast microscope.

Appendix 2: Fatty Acid Metabolism and the Isolated Muscle Preparation

Rat hindlimb perfusions, and the incubated intact muscle preparation are the primary methods of choice for investigating skeletal muscle FA metabolism in rodents (42). The *in vitro* incubation of an isolated intact muscle preparation (rodent and human) is the primary technique utilized in this thesis. In the late 1960's Moorthy and Gould (237) first began to use the rat soleus muscle because it was easily exposed and had a well-defined tendon, which allowed for easier removal while minimizing damage to the muscle. In addition the presence of a long tendon prevented the muscle from "leaking" cytosolic components therefore promoting muscle viability. More recently this technique has been adapted for use in human rectus abdominus tissue by Dohm et al. (98). The isolated muscle preparation allows for precise control of hormonal and substrate concentrations and also has the advantage of eliminating problems associated with perfusion flow rates as oxygen and substrates enter the muscle exclusively via diffusion from the incubation medium. However, this can also be a potential disadvantage if incubation conditions are not controlled properly, because diffusion of substances may be inadequate and muscle viability (as determined by the concentration of ATP, PCr, and muscle glycogen) may become compromised. In order to ensure adequate and homogeneous substrate diffusion throughout the entire muscle a number of factors must be controlled. First, excision of the muscle must be rapid as prolonged periods without oxygen may increase the area of the hypoxic core. Secondly, incubation buffers must be saturated with O₂. Therefore, the primary problem with the *in vitro* preparation is not the content of oxygen within the incubation medium but instead the ability of oxygen to diffuse across all muscle fibres at

a rate greater than or equal to the metabolic rate of the muscle. Metabolic rate has been shown to be dependent on the temperature of the incubation medium. Segal and Faulkner (298) demonstrated that muscle viability was inversely related to muscle thickness and the temperature of the incubation medium; that is, as muscle thickness increases temperature must be reduced in order to maintain muscle viability. At a temperature of 30°C (as utilized in all experiments in this thesis) muscle radius cannot exceed 1.55 mm, which corresponds to a rat soleus muscle weight of approximately 25-30 mg. An adult rat soleus muscle weights approximately 100 mg; therefore in order to reduce the diffusion distance; small muscle strips are removed from the soleus muscle.

Fatty acids derived from the plasma, represents the major source of fuel for resting skeletal muscle. While this has been recognized for several decades, knowledge regarding the uptake of FA and their subsequent metabolism (esterification or oxidation) is limited, especially when compared with our knowledge of carbohydrate metabolism. This is partly due to the fact that plasma FA are in very low concentrations in the circulation and are so energy dense that small errors in their concentration represent large changes in rates of ATP provision. Furthermore, the role of intramuscular TG as a fuel source in skeletal muscle, in both humans and rodents, has been controversial and difficult to demonstrate due to technical difficulties in measuring muscle TG and the inherent variability in its content (313, 358). This is because intramuscular TG deposition is not uniform within muscle fibres or across fibres types and also because of the high energy density of contaminating intermyocytal adipocytes. In humans,

Wendling et al. (358) reported that due to the inherent high variability between muscle biopsy samples only changes greater than 24% in the intramuscular TG pool could be detected. While in rats, the variation of intramuscular TG within a given muscle sample is acceptable (5-13%, see Table 12 and (313)), the variation between left and right legs has been shown to be as high as (20-50%) (313). The combination of these factors combined with the constant turnover of the intramuscular TG pool (simultaneous hydrolysis and esterification) contribute to the high variability in intramuscular TG measurements which can obscure attempts to accurately measure changes.

Table 12. Intramuscular TG variability in aliquots of rat red (RG) and white gastrocnemius (WG) muscle

	1WG	2WG	3 WG	1RG	2RG
Aliquot #1	7.8	20.4	42.1	9.8	7.2
Aliquot #2	8.2	24.0	47.0	11.5	6.7
Aliquot #3	8.1	17.9	46.0	12.5	7
Aliquot #4	9.7	18.4			6.0
Mean	8.5	20.2	45.0	11.3	6.7
STDEV	0.9	2.8	2.6	1.4	0.5
CV	10.1	13.7	5.7	12.1	7.8

Mean CV = 9.9 ∨ 3.2 (SEM)

Appendix 3: The Pulse-Chase Technique

Due to the difficulties outlined above an alternative approach to measuring FA metabolism is through the use of radioisotopic tracers. In most cases an isolated skeletal muscle is incubated with a ^{14}C labeled FA (usually either oleate or palmitate) and a known concentration of “cold” or unlabeled FA. Fatty acid oxidation can then be measured by the appearance of $^{14}\text{CO}_2$ while FA esterification into the various lipid pools is determined by extracting the lipids and measuring the radioactivity. The use of the radioisotope provides superior levels of sensitivity (nanomolar) for measuring FA oxidation and esterification when compared to indirect calorimetry and traditional enzymatic techniques, respectively. The use of a single radioisotope does not allow the user to differentiate between exogenous and endogenous sources of FA or the ability to simultaneously evaluate TG synthesis and hydrolysis. In 1991 in isolated perfused rat hearts, Saddik and Lopaschuk (284) extended the single tracer technique to simultaneously evaluate synthesis and breakdown of the TG pool while also measuring endogenous oxidation using a dual-tracer, pulse-chase technique. By first “pulsing” the hearts with ^{14}C -palmitate, they pre-labeled the endogenous pools (e.g. TG, DG, PL). The hearts were then “chased” with ^3H -palmitate. Simultaneous measurement of ^3H palmitate incorporation in the various lipid pool or oxidation of $^3\text{H}_2\text{O}$, as well as ^{14}C -labeled TG hydrolysis to $^{14}\text{CO}_2$ provides information with respect to intramuscular hydrolysis and synthesis as well as oxidation of endogenous and exogenous FA. Dyck et al. (104) were the first to utilize the pulse-chase technique in isolated skeletal muscle. The use of the radioisotopes provided superior levels of sensitivity to measure FA esterification and

hydrolysis as well as the oxidation of both endogenous and exogenous lipids and because the muscle is isolated it has the advantage of permitting precise control of hormone and substrate concentrations.

Despite the many advantages of the pulse-chase technique there are also significant limitations and assumptions associated with the technique (104). In order to determine the rate of TG hydrolysis it is necessary to know whether the labeled FA incorporated during the pulse is homogeneously distributed and representative of the entire pool, or whether the labeled portion represents only a small subpool with a relatively rapid turnover (i.e. last in...first out phenomenon). In preliminary studies it was determined that only ~10% of the total TG pool was labeled during a 3-h incubation (~400 nmol/g wet wt out of the total pool size of 4.1 μ mol/g wet wt.). This is in contrast to work in contracting heart where ~40% of the total TG pool was labeled (284). In initial experiments while there was significant TG hydrolysis (i.e. loss of label) (-32%), there was no detectable decrease in total TG content as measured using traditional enzymatic techniques. On the basis of this data, the incorporated ^{14}C palmitate does not appear to "track" the entire TG pool, but rather a very small sub pool with and apparently more rapid turnover. Therefore, care must be taken when extrapolating the changes in TG hydrolysis to the turnover of the entire TG pool. It is important to note that during muscle contraction the loss of pre-labeled palmitate parallels the decrease in intramuscular TG content (102). A second limitation of the present technique is that only the net disappearance of the labeled palmitate from within a given pool can be traced and not the movement of labeled palmitate between pools. Therefore, it is possible that a

small loss of pre-labeled palmitate from, for example the TG pool may, be replaced by labeled palmitate from other lipid pools e.g. DG and PL. This turnover between pools could mask the cumulative loss from the TG pool. Lastly, it is possible that a significant contribution of the endogenous oxidation may come from “trapped” nonincorporated palmitate that was not eliminated during the wash. It has previously been demonstrated that 5-8 nmol/g wet wt. of palmitate remained in the muscles at the end of the wash (representing ~5% of the trapped palmitate at the start of the wash), which, if all oxidized, would lead to a slight overestimation of endogenous palmitate oxidation. Despite the disadvantages outlined above which may limit the accuracy of the technique in measuring absolute rates of TG breakdown and endogenous FA oxidation the superior sensitivity of the technique combined with the ability to precisely control substrate and hormonal concentrations makes it an appropriate technique to measure the relative changes of leptin treatment on human and rodent skeletal muscle FA metabolism.

Appendix 4: Quality Control Experiments.

To determine that the SOL muscle preparation used in this thesis was an appropriate model, muscle strips were rapidly frozen in liquid N₂ after excision (control), or after 20, 90 or 180 min of incubation under the same conditions in which pulse-chase experiments were conducted. Concentrations of ATP and PCr were then measured spectrophotometrically (27). The results of these experiments are summarized in Table 13. Secondly, to determine whether the incorporation of both [9,10- ³H] and [1-¹⁴C] palmitate labels was linear over the entire incubation protocol we measured the incorporation of [9,10- ³H] and [1-¹⁴C] palmitate into TG (Fig. 24) pools and the production of ¹⁴CO₂ and ³H₂O (Fig. 25) at rest, after 60, 120 and 180 min of incubation. Similar experiments were conducted in human rectus abdominus muscle strips (Table 11, Fig. 27 and Fig. 28). The results of these experiments indicate that muscle viability is preserved under the incubation conditions employed and that the use of both [9,10- ³H] and [1-¹⁴C] palmitate results in uniform labeling and linear rates of incorporation into the various lipid pools over the entire incubation protocol. In addition, exogenous oxidation measured by the production of both ¹⁴CO₂ and ³H₂O also demonstrate that oxidation is linear over the entire incubation period and rates of palmitate oxidation are nearly identical irrespective of the label used.

In order to determine that we were capturing all ¹⁴CO₂ produced, in a separate experiment, complete recovery of [¹⁴C] bicarbonate ($98.3 \pm 3\%$ $n=6$) was confirmed. In another experiment we determined the recovery rate of ¹⁴C and ³H palmitate once spotted

on thin layer chromatography plates and were able to demonstrate a $(94.5 \pm 5\%, n=4)$ recovery when the entire plate was scraped and counted.

Table 13. Muscle viability of rat soleus muscle incubated at 30°C

	Controls	20 min	90 min	180 min
ATP	19.56 $\pm$ 0.75	17.34 $\pm$ 0.97	16.11 $\pm$ 0.96	15.36 $\pm$ 0.86
PCr	44.23 $\pm$ 2.59	35.65 $\pm$ 3.15	40.94 $\pm$ 1.92	40.33 $\pm$ 4.42

Values are means $\pm$ SE and are reported as :mol/g dry wt: $n=12$ (controls), $n=11$ (20 min), $n=9$ (90 min), $n=9$ (180 min).

Figure 25. TG esterification of ^{14}C and ^3H palmitate over 180 min in resting rodent soleus muscle

Figure 26. Palmitate Oxidation as measured by the production of $^{14}\text{CO}_2$ and $^3\text{H}_2\text{O}$ over 180 min in resting rat soleus muscle

Figure 27. Triacylglycerol esterification of ^{14}C and ^3H palmitate over 180 min in resting human rectus abdominus muscle strips

Figure 28. Palmitate oxidation as measured by the production of $^3\text{H}_2\text{O}$ and $^{14}\text{CO}_2$ over 180 min in resting human rectus abdominus muscle strips