

DIRECT AND INDIRECT EFFECTS OF LEPTIN ON PREADIPOCYTE
DEVELOPMENT

by

COURTNEY BLAIR WAGONER

(Under the direction of Ruth Harris)

ABSTRACT

Leptin has been shown to reduce body fat *in vivo*. Leptin could regulate adipose mass by inhibiting preadipocyte replication, commitment to the adipocyte lineage, and/or lipid accumulation. We determined the effect of leptin, serum from leptin infused rats, and adipose conditioned media from leptin infused rats on preadipocyte proliferation and differentiation in a primary culture of cells from male Sprague-Dawley rat adipose tissue. Leptin (50 ng/ml) stimulated proliferation of preadipocytes, but 250 and 500 ng leptin/ml inhibited proliferation of both preadipocyte and stromal vascular fractions, measured by ³H-thymidine incorporation. Serum (0.25% by volume) from leptin infused rats inhibited proliferation of preadipocyte ($p < 0.06$) and stromal vascular ($p < 0.05$) fractions. Conditioned media (30% by volume) had no effect on proliferation of either cell fraction. None of the treatments changed preadipocyte differentiation. These results suggest that leptin could inhibit preadipocyte proliferation by modifying release of a factor from tissue other than adipose tissue.

INDEX WORDS: leptin, adipogenesis, preadipocyte, proliferation,
differentiation

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CHAPTER 1

INTRODUCTION

Obesity is a condition of increased body fat mass resulting from an increase in the number and/or size of fat cells [1]. In 2002, nearly one billion people worldwide were overweight or obese [2]. Overweight and obesity are a huge problem in the United States, with 65% of the population being overweight and 30% obese [2]. Several factors increase a person's risk of developing obesity, such as decreased physical activity, high fat and/or high calorie diets, behavior and genetics [3]. Obesity is associated with an increased risk for other health complications, such as arthritis, type II diabetes, high blood pressure, stroke and certain types of cancer (www.cdc.gov). In 2000, health care costs associated with obesity reached \$117 billion in the United States alone (www.surgeongeneral.gov).

Researchers are continuously trying to find ways to prevent and/or cure obesity in order to decrease the negative health consequences and increased health care costs associated with the disease. The answer seems simple: balance energy intake and expenditure [3]. However, researchers have discovered that obesity is much more complex, and the exact mechanisms leading to the development of obesity are not fully understood [4]. Several treatment methods have been studied extensively, such as diet therapy, physical activity, behavioral treatment, surgery and pharmacotherapy [5]. Although each treatment method shows promise in some individuals, none have been able to treat or prevent overweight and obesity in all individuals [5]. Additionally, most people who lose weight regain it within 3-5 years [3,5].

It is important to understand the physiological mechanisms leading to obesity in order to prevent and/or treat the disease. In rodents, a feedback regulatory loop responsible for maintaining a constant body weight has been established [1]. The lipostatic theory suggests that there is a product of fat metabolism circulating in the plasma that interacts with the hypothalamus to control the size of body fat depots. Early parabiosis experiments support the lipostatic theory suggesting [6] that a circulating factor is released from fat in proportion to the amount of fat in the body, and this factor acts on the hypothalamus to decrease food intake [7]. In 1994, a circulating factor, leptin, was discovered [6]. Leptin is secreted by fat cells and circulating concentrations of leptin correlate with fat mass, suggesting that it might function as the circulating factor identified by parabiosis studies [8]. In animal studies leptin decreases food intake and body weight, but most overweight and obese humans have high levels of circulating leptin, implying that they are resistant to the effects of leptin [9-10].

Initially, it was thought that leptin exerted its effects by binding to central receptors to decrease food intake and body weight and increase energy expenditure. Later parabiosis studies showed that intraperitoneal (ip) leptin administration decreases body fat in lean and obese parabionts, but only decreases food intake in the obese partner [11]. Additionally, Bowen et al. showed that peripheral infusions of 10 µg/day of leptin decrease body fat, but not food intake, which implies that the loss of fat is not entirely due to decreased food intake [12]. Other ways that peripheral leptin administration could decrease body fat are by activating the sympathetic nervous system to increase energy expenditure [13] or by altering adipocyte metabolism so that energy is diverted to other tissues [14-16].

Leptin has been shown to increase lipolysis *in vitro* and *in vivo* and inhibit adipocyte lipogenesis *in vitro* [14-16]. Stimulation of lipolysis occurs with much lower leptin concentrations *in vivo* versus *in vitro*, suggesting that leptin may activate another factor that is actually responsible for stimulation of lipolysis [14-16]. In addition, leptin could act directly on fat cells to alter adipocyte development by inhibiting preadipocyte proliferation (increase in cell number) and/or differentiation (lipid filling). Two studies have examined the effect of leptin on preadipocyte proliferation [17-18]. One study used a primary culture of rat preadipocytes and showed that 160 ng/ml murine leptin increased proliferation [17], but stromal vascular cells were not separated from adipocytes, so it is not certain whether preadipocytes, stromal vascular cells, or both were responsible for the observed increase in proliferation. The other study used a primary culture of pig preadipocytes and showed that 1,000 ng/ml porcine leptin increased preadipocyte proliferation [18]. The different sources of preadipocytes and leptin could account for the different doses of leptin required to see an effect on proliferation. Three studies have examined the effect of leptin on preadipocyte differentiation *in vitro*, and all three produced conflicting results [17-19].

This thesis examines whether leptin is able to modify preadipocyte development, and if so, whether the effect is direct or indirect. The experiments in this paper tested the effect of leptin alone, serum from leptin infused animals, and adipose conditioned media from infused animals on preadipocyte proliferation and differentiation in a primary culture of stromal vascular cells obtained from male Sprague-Dawley rats. ³H-thymidine incorporation was used as a measure of proliferation, and GPDH activity was used to measure differentiation.

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CHAPTER 2

LITERATURE REVIEW

LEPTIN

Obesity

Obesity is a condition of increased body fat mass resulting from an increase in the number and/or size of fat cells [1]. In 2002, nearly 1 billion people were overweight or obese, making obesity the most common nutritional disorder in Western societies [2]. This number keeps rising, signifying obesity as a huge problem, especially in America. In 2002, 65 % of American adults were overweight, 30.5% obese, and 4.9% morbidly obese [3]. Additionally, almost 16% of children aged 6-19 were overweight, representing a 4.5 % increase from 1994 [3, www.cdc.gov].

Obesity increases the risk of about 30 medical conditions, ranging from carpal tunnel syndrome, daytime sleepiness and low back pain to much more severe conditions, such as arthritis, type II diabetes, high blood pressure, stroke and certain types of cancer (www.obesity.org). In addition, obesity is a strong independent risk factor for developing heart failure [2]. High blood pressure, which leads to strokes, and cervical cancer are two times more common in obese than non-obese people (www.cancer.org). Three hundred thousand Americans die every year from obesity-related health problems [4].

It is known that obesity results from a chronic imbalance between energy intake and expenditure [1]. Neural activity and hormones are known to regulate body weight, but the exact mechanisms that lead to the development of obesity are not fully understood

[1, 5-9]. One hormone that is known to affect obesity is insulin. After a meal, glucose levels rise and insulin secretion is stimulated to clear the blood of glucose and to store it in the form of glycogen in muscle and liver and as triglycerides in adipose tissue [9]. When insulin secretion is low (type I diabetes) or when cells are resistant to the effects of insulin (insulin resistance and/or type II diabetes), regulatory hormones are released which create a shift from carbohydrate metabolism to fat metabolism, resulting in increased gluconeogenesis and fatty acid release from adipose tissue [9]. Chronic exposure to high levels of free fatty acids results in increased triglyceride content in adipose tissue and other organs [9]. Both hyperglycemia and high levels of free fatty acids are characteristics of obesity [9-10].

A high fat diet, which oftentimes leads to obesity, has been shown to decrease the number of insulin receptors on target tissues and increase insulin resistance, while a reduction in fat intake can reverse insulin insensitivity in female rats [11-12]. There are conflicting results on the relationship between low fat diet and improved insulin sensitivity in humans [13-14]. Additionally, most obese humans have high levels of insulin and reduced insulin sensitivity and more than 80% of people with type II diabetes are obese [11,15].

The exact mechanism by which obesity, insulin resistance and type II diabetes are related is still unclear. Current theories suggest that the excess fatty acids, especially saturated fatty acids, present in obesity accumulate in tissues and disrupt insulin signaling pathways leading to insulin insensitivity and resistance [16]. Additionally, adipose tissue in male mice remains insulin sensitive when the liver is resistant [17]. Therefore, insulin

resistance leads to obesity because the excess glucose and fatty acids are stored in insulin sensitive adipose tissue [17].

Body fat is also regulated by neural activity [5-8, 18-21]. The sympathetic nervous system (SNS) innervates white adipose tissue (WAT) and brown adipose tissue (BAT) as evidenced by tract tracing studies showing an increase in labeled neurons in the brain stem, midbrain, hypothalamus, forebrain and spinal cord following injection of a retrograde fluorescent tracer into epididymal(e) WAT, inguinal(i) WAT and interscapular BAT [5-7]. SNS activation increases lipolysis and decreases fat cell number in WAT [7-8], while increasing thermogenesis in BAT [18]. Fat cells contain four subtypes of adrenoceptors (AR), α_2 and β_{1-3} [19]. All three β -ARs are expressed in white and brown adipocytes in rodents [20]. Activation of the β -ARs by catecholamines stimulate lipolysis [19-21]. In contrast, surgical denervation of iWAT and retroperitoneal(r) WAT in hamsters, which disrupts sympathetic and sensory innervation to these tissues, significantly increases fat mass and fat cell number without affecting fat cell size [7, 22]. This implies that SNS innervation into adipose tissue is essential for body weight regulation.

Leptin and Obesity

In mice, mutations in the diabetes (db) and obese (ob) genes produce similar metabolic abnormalities, including obesity, infertility, hyperphagia, insulin resistance and reduced number of insulin receptors [23]. These genes are located on separate chromosomes, which mean that obesity and diabetes are probably not caused by the same primary lesion, but the similarities in phenotype imply that they impact the same metabolic pathways [23].

The lipostatic theory suggests that there is a product of fat metabolism circulating in the plasma that interacts with the hypothalamus to control the size of the body fat depots [24]. It has been shown that the level of ob expression correlates with fat pad weight in the inguinal, ovarian, retroperitoneal and perirenal fat pads [25]. Therefore, it has been hypothesized that leptin is the circulating signal informing the brain of the size of body fat stores [26].

This theory is supported by parabiosis experiments from Coleman in 1973 [27]. Parabiosis of diabetes (db/db) mice with normal or obese (ob/ob) mice resulted in death of the normal or ob/ob mice due to starvation. These results imply that db/db mice produce a satiety factor, which normal and ob/ob mice can respond to, but db/db cannot. The union between two db/db mice resulted in no change because neither one could respond to the factor, so both continued to eat [27]. Harris [28] showed that lean and obese partners (ob/ob) of a lean-obese parabiont both lost body fat compared to lean-lean and ob/ob-ob/ob controls. This loss of body fat was accompanied by a 25% reduction in food intake by the ob/ob partner, but not the lean partner. Since parabiosis experiments connect the circulatory system of the animals, it is known that the satiety factor is blood borne. In 1994, Zhang et al [24] found that the normal mouse ob gene produces a 16 kDa protein, now known as leptin, and that mutations in this gene cause severe obesity. This implies that leptin is the circulating satiety factor.

Leptin circulates in plasma [24] and intraperitoneal (i.p.) administration of leptin to obese mice results not only in a dose dependent decrease in body fat, but also an increase in lean mass [29]. Loss of fat mass cannot be completely attributed to decreased food intake as Bowen et al. [30] showed that peripheral infusions of 10 µg/day of leptin

decrease body fat, but not food intake. I.p. administration of leptin to ob/ob mice decreases food intake [26], while direct injections of leptin into the brain also increases energy expenditure, which contributes to the reduction of body fat [26,31].

The Leptin Receptor

Leptin initiates biological responses by binding to its receptor, Ob-R, which is found in the brain and peripheral tissues [32]. Ob-R is a class 1 cytokine receptor [33] and has five isoforms: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re [33]. Three isoforms (Ob-Ra, Ob-Rc and Ob-Rd) have short intracellular domains [34]. Ob-Rb has a long intracellular domain and is important for initiating intracellular signal transduction [34,35]. Ob-Rb is expressed at high levels in the hypothalamus, cerebellum and beta cells, and at lower levels in most other tissues, including adipose tissue [35]. Ob-Ra is highly expressed in the kidney, lung, intestine, heart, testes, choroid plexus, brain microvessels, and adipose tissue [35] and has been hypothesized to transport leptin from the periphery to the brain [35].

Since direct injections of leptin into the brain decrease appetite and body fat [26], it was originally thought that leptin exerted its effects by binding to receptors in the central nervous system [32]. More recent studies have shown that leptin may act on leptin receptors in peripheral tissues or stimulate another pathway to reduce body fat. Harris et al. [36] peripherally infused increasing concentrations of leptin (0, 1, 2, 5, 10, or 42 $\mu\text{g/d}$) into lean and obese C57Bl/6J mice for seven days. This resulted in a dose dependent increase in circulating leptin concentrations up to the 10 $\mu\text{g/day}$ leptin level in both lean and obese mice. In addition, ob/ob mice had significantly lower food intake and body weight than controls at all leptin levels higher than 1 $\mu\text{g/day}$. The lean mice were less

sensitive to leptin and had reduced body fat without a significant inhibition of food intake, implying that decreased energy intake may not be essential for leptin to have its effect on body fat in normal animals.

Leptin Resistance

Leptin resistance occurs when leptin signaling is disrupted, leading to energy imbalance [30]. In this state, leptin is not able to decrease body weight despite increased concentration of the protein [30]. db/db mice lack functional leptin receptors and have excessive amounts of circulating leptin, but they are obese, implying a role for leptin in the regulation of body fat [37]. Additionally, diet induced obesity (DIO) can cause leptin resistance [30, 38-39]. DIO initially induces leptin resistance in response to peripherally administered leptin, which is probably caused by a defect in leptin transport across the blood-brain barrier. [38-40]. After time, sensitivity to centrally administered leptin is also reduced, and the decreased sensitivity seems to be specific to the arcuate nucleus [41].

In humans, leptin levels are positively correlated with BMI due to increased production and decreased clearance of the protein in overweight and obese individuals [42-43]. Unlike rodents, there are very few cases of leptin deficiency in human obesity [42-44]. Administration of leptin to obese humans on a calorie reduced diet decreases fat mass, implying that obese humans are not totally leptin resistant, but require more leptin than lean individuals to produce weight loss [44].

Central actions of leptin

In rodents, the presence of a feedback regulatory loop responsible for maintaining a constant body weight has been established [1]. This feedback loop involves three components: a peripheral sensor to monitor the amount of fat in the body, hypothalamic

centers that receive information from the peripheral sensor, and effector systems that control energy intake and expenditure [1]. Leptin acts as a peripheral sensor because it is released by fat cells in proportion to the amount of fat present, and it reduces body fat by decreasing food intake and increasing energy expenditure [31, 37, 45]. Circulating leptin crosses the blood brain barrier (BBB) and binds to receptors in the brain [46]. Experimentally, intracerebroventricular (ICV) injections of leptin decrease food intake and body weight and increase energy expenditure [26, 31, 47].

One way that leptin decreases food intake is by downregulating neuropeptides that stimulate feeding. Neuropeptide Y (NPY) and Agouti-related protein (AGRP) are neuropeptides involved in feeding regulation [48]. NPY is a potent orexigenic peptide, while AGRP is an endogenous antagonist of the melanocortin system and inhibits the appetite suppressing activity of melanocortin agonists, such as α -MSH [49]. These neuropeptides are coexpressed in neurons in the arcuate nucleus (ARC) of the hypothalamus, and are released in the PVN [49], where they act to stimulate food intake but are released in various other hypothalamic nuclei [48, 50]. Fasting increases expression of NPY and AGRP, while feeding decreases their expression [51-52].

Leptin downregulates NPY and AGRP expression [1]. Constant subcutaneous infusion of leptin in mice or rats or long-term exposure to ICV leptin in mice decreases ARC NPY expression and inhibits the rise in NPY mRNA during fasting [53-54]. Additionally, ip injections of leptin decrease AGRP expression in mice [55] and *in vitro* incubation of perfused hypothalamic slices with leptin decreases the release of AGRP [56].

Overall, leptin acts through the central nervous system to decrease food intake, but as discussed below, leptin also activates the SNS, which stimulates energy expenditure.

Leptin, the Sympathetic Nervous System and Adipose Tissue

Leptin acts through the central nervous system to activate the SNS, as illustrated by increased thermogenesis in BAT in rats infused with leptin [57]. Haynes et al. [58] measured sympathetic nerve activity (SNA) stimulated by subcutaneous leptin infusion in rats with and without functional leptin receptors. Rats with functional leptin receptors had a dose dependent increase in SNA to BAT and the kidney, hindlimb, and adrenal gland, whereas rats without leptin receptors had no increase in SNA to any of the tissues measured.

It is uncertain whether leptin stimulates SNS outflow to WAT. Leptin increases lipolysis in WAT [59-61], but it is unclear whether the increase is due to a direct action of leptin on adipocytes or activation of the SNS. Leptin injection into eWAT of male Wistar rats increased afferent and efferent nerve activity in WAT, implying that leptin sensors in WAT activate the SNS, and in turn, increase SNS outflow to WAT [62-63]. Lastly, leptin requires norepinephrine to regulate its own expression in WAT and UCP-1 expression in BAT, suggesting that leptin acts indirectly, through stimulation of the sympathetic nervous system, to control its own expression in WAT and increase UCP-1 expression in BAT [64].

Leptin and Adipocyte Metabolism

It is known that leptin acts directly on the hypothalamus and brainstem to decrease appetite and increase energy expenditure, which in turn, decreases body fat [31,

48]. Studies have also shown that it is possible for leptin to act directly on adipose tissue since leptin receptors are found on adipocytes [35]. Leptin could reduce body fat either by increasing lipolysis (breaking down fat into free fatty acids and glycerol) and/or inhibiting lipogenesis (triglyceride synthesis) [59-61, 65-66].

Leptin has been found to increase lipolysis *in vitro* [59-61] and *in vivo* [65] and decrease lipogenesis *in vitro* [61,66]. Addition of leptin to isolated adipocytes from ob/ob, lean and db/db mice increases lipolysis of the ob/ob and lean adipocytes as measured by glycerol release [59]. Lipolysis is greater in adipocytes from ob/ob mice compared to lean mice, with maximal stimulation at 1.25×10^{-5} M (200,000 ng/ml) leptin [59]. Addition of leptin to adipocytes from db/db mice lacking the long-form leptin receptor does not increase lipolysis [59]. Leptin (20 ng/ml, 1.25×10^{-9} M) increases release of glycerol, but not free fatty acids, from primary adipocytes collected from lean rats while 100 nM epinephrine increases the release of free fatty acids and glycerol [60]. This implies that in addition to promoting lipolysis, leptin increases intracellular fatty acid oxidation in the adipocyte so that only glycerol is released.

In vivo, intraperitoneal injections of leptin (0.1, 1.0, and 10.0 mg/kg body weight) in ob/ob, db/db and lean mice result in a dose dependent increase in lipolysis in ob/ob mice, as measured by glycerol release [65]. The increase is significant at the 1.0 and 10.0 mg/kg body weight leptin concentrations [65]. In lean mice, 10 mg/kg body weight leptin significantly increased lipolysis [65]. There was no effect of leptin on lipolysis in db/db mice [65].

Leptin decreases expression of the lipogenic enzymes, fatty acid synthetase (FAS) and acetyl CoA carboxylase (ACC), and increases expression of fatty acid

oxidation enzymes, acetyl CoA oxidase (ACO) and carnitine palmitoyl transferase-1 (CPT-1) *in vitro* [61,66]. 20 ng/ml leptin decreases FAS and increases ACO and CPT-1 in adipocytes isolated from lean Zucker rats [61]. Leptin (200, 400 and 600 ng/ml) added to post-confluent 30A5 adipocytes results in a dose dependent suppression of ACC activity [66]. These *in vitro* studies illustrate that leptin decreases fatty acid synthesis by acting directly on adipocytes, and that neither the brain, nor signals from the brain, are involved in the suppression of lipogenesis due to leptin.

In addition to increasing lipolysis and decreasing lipogenesis, leptin may affect adipocyte growth. Three studies have examined the effect of leptin on preadipocyte differentiation (lipid filling), but the results are contradictory [67-69]. Two studies have shown that leptin increases preadipocyte proliferation (increased cell number) [67, 69]. This is discussed in more detail below.

Leptin Interactions with Hormones, Cytokines, and Other Factors

Insulin

Adipocytes are highly sensitive to insulin, which is a mitogenic factor that may indirectly influence adipocyte growth [17]. The relationship between insulin and leptin is complex and not fully understood. Insulin has been shown to stimulate leptin expression and secretion in cell lines [70] and primary cell and tissue cultures [71]. Leptin also may act to decrease insulin sensitivity in adipose tissue, while increasing insulin sensitivity in liver and muscles [72]. Additionally, leptin may decrease insulin secretion in pancreatic β cells [73].

In the presence of leptin, metabolic activities associated with insulin such as stimulation of glucose transport, glycogen synthase, lipogenesis and protein synthesis are

inhibited in primary rat adipocytes [74]. The effects are seen at physiologic leptin levels of 1 nM (16ng/ml) and insulin concentrations from 5-10 nM, and increase dose dependently with almost complete inhibition at >30 nM leptin (480 ng/ml) [74]. The same study showed that leptin decreases sensitivity of primary rat adipocytes to insulin over several hours, but insensitivity is fully reversible a few hours after leptin removal as measured by basal and insulin-stimulated glucose transport at various time points after leptin removal [74].

In vivo, leptin has been shown to increase insulin sensitivity acutely in mice fed a high fat diet, but not over long periods of time [75]. Leptin has been shown to have a time and tissue specific effect on insulin action *in vivo* [73]. In lean mice, leptin does not change glucose stimulated insulin release after acute exposure (2 hr), but inhibits whole body glucose clearance, which implies that leptin reduces insulin action [73]. Chronic infusion (7 days) of leptin decreases glucose stimulated insulin release without severe hyperglycemia, which implies improved insulin sensitivity [73]. In response to chronic leptin infusion, insulin responsiveness increased in muscle and liver tissue, but adipose tissue was insulin resistant, suggesting that nutrients are taken to muscle and liver tissue instead of enlarging adipose tissue [73].

TNF- α

TNF- α is a cytokine produced by macrophages, adipose tissue and many other tissues [76-77]. TNF- α mRNA is expressed in subcutaneous adipose tissue, but there are conflicting results whether the majority of TNF- α is secreted from adipocytes or non-adipose cells in adipose tissue [76]. Both forms of the TNF- α receptor, TNFR-I and II, are found in adipose tissue [78].

Like leptin, TNF- α decreases food intake and modulates lipid metabolism [79]. Additionally, both leptin and TNF- α regulate insulin sensitivity, but TNF- α induces insulin resistance whereas leptin improves insulin sensitivity [73, 76, 80]. Additionally, TNF- α is increased in adipose tissue in human and rodent models of obesity, but circulating TNF- α and obesity do not seem to be correlated [81], suggesting that TNF- α acts as an autocrine/paracrine factor in adipose tissue. Low levels of TNF- α (1 and 2.5 ng/ml) increase insulin resistance in cell lines *in vitro*, but the inhibition can be reversed within 3 days after TNF- α removal [79]. In humans, TNF- α expression in adipose tissue is highly correlated with fasting plasma insulin, and weight loss decreases TNF- α expression and serum insulin, supporting the notion that TNF- α induces insulin resistance [76].

Since leptin and TNF- α are both cytokines and have many similar functions, it would not be unrealistic that the two work together to reduce fat mass. TNF- α stimulates leptin levels *in vivo*, but not *in vitro* [81]. *In vivo*, intravenous TNF- α administration (0.1-1 μ g) to C57BL/6J male mice increases plasma leptin levels and leptin expression in adipose tissue [81]. Additionally, obese mice lacking TNF- α have decreased circulating leptin levels [81].

In vitro, addition of 2.5 nM TNF- α to 3T3-L1 adipocytes increases leptin initially, but is followed by a sharp decrease in production over the next 18 hours [81]. The varying results *in vitro* and *in vivo* could be explained by the fact that *in vitro* culture systems do not contain all the factors found *in vivo* or that an essential cofactor/nutrient is depleted in the culture.

While TNF- α seems to increase leptin, leptin decreases TNF- α expression *in vivo* [82]. Leptin (5 μ g/day) administered for 5 days intracerebroventricularly (ICV) decreases TNF- α expression in adipose tissue [82]. Therefore, leptin and TNF- α probably is involved in a feedback loop where TNF- α stimulates leptin which, in turn, decreases TNF- α . Additionally, decreased fat mass probably is responsible for reduced TNF- α expression in adipose tissue following leptin administration.

PPAR γ

PPAR- γ is a transcription factor found mostly in adipose tissue [82-83] and is a positive regulator of adipocyte differentiation [83]. PPAR- γ interacts with both leptin and TNF- α [82]. It has been shown to inhibit leptin gene expression *in vivo* and *in vitro* [84]. ICV leptin administration (5 μ g/day) increases PPAR- γ expression [82]. ICV leptin administration decreases fat, so it is surprising that PPAR- γ expression would increase since PPAR- γ is a positive regulator of adipocyte differentiation.

α -MSH

Pro-opiomelanocortin (POMC) neurons are found mainly in the arcuate nucleus (ARC) of the hypothalamus, as well as other parts of the hypothalamus and in the periphery [85]. The POMC gene produces α -melanocyte stimulating hormone (α -MSH) [85]. α -MSH acts on melanocortin receptor subtypes MC3-R and MC4-R to regulate food intake and control energy balance [85-86]. It is now known that α -MSH not only acts in the brain to reduce food intake, but also acts in the periphery to reduce body weight [85-87]. Peripheral injections of an α -MSH analog into ob/ob mice results in significant weight loss [87].

Recent studies have shown that leptin and α -MSH control each other's expression and secretion [85-86]. POMC neurons in ARC express the leptin receptor, Ob-R, and adipose tissue contains the MC4 receptor [85,88]. Circulating α -MSH levels are lower in ob/ob mice lacking leptin than in lean controls [87]. Additionally, intraperitoneal administration of leptin to these mice increases circulating α -MSH two-fold [87]. α -MSH has been shown to decrease leptin secretion and expression *in vitro* [86]. Therefore, it is possible that there is a feedback loop between leptin and α -MSH, with leptin increasing α -MSH secretion and α -MSH downregulating leptin expression and secretion [85].

IL-15

Interleukin (IL)-15 is a cytokine with many functions, including T-cell stimulation, enhancement of natural killer (NK) cells, muscle protein accretion, lymphoid homeostasis, and possibly reduction of adipose mass [89-90]. IL-15 is expressed in a variety of tissues including skeletal muscle, placenta, kidney, lung, heart, and adipose tissue [89-90]. The IL-15 receptor has three subunits, α , β , and γ c, which are all expressed in adipose tissue of lean rats [89]. However, the γ c subunit, which is responsible for signaling, is markedly decreased in obese rats [89]. These metabolic responses to IL-15 are very similar to those caused by leptin administration.

Lean (+/?) Zucker rats, as well as lean (+/+) and obese (ob/ob) C57BL/6 mice showed significantly reduced dorsal white adipose tissue mass when given 100 mg/kg body weight of IL-15 subcutaneously for seven days [89]. This loss of fat was not accompanied by decreased food intake. Additionally, obese (fa/fa) rats did not lose fat mass or decrease food intake in response to IL-15 [89]. Male Wistar rats receiving IL-15

subcutaneously at a dose of 100µg/kg body weight every day for seven days had significantly lower body weights and rates of protein degradation than control rats [91-92]. Additionally, IL-15 decreases circulating triacylglycerol levels [92].

In vitro treatment of porcine adipocytes with 50 and 100 ng/ml of IL-15 had a dose and time dependent effect on lipolysis, with the greatest stimulation occurring 2 hours after exposure to IL-15 [90]. Using selective inhibitors of various signaling pathways, it was found that IL-15 acts through Protein Kinase A and Jak-Stat pathways to stimulate lipolysis [90]. IL-15 also reduces the incorporation of [U-¹⁴C] glucose into fatty acids, which implies that IL-15 decreases lipogenesis [90]. Administration of subcutaneous IL-15 to male Wistar rats decreases circulating leptin concentrations [92]. In addition, leptin has been shown to increase IL-15 activation of NK cells [93]. It is possible that leptin activates IL-15 and IL-15, in turn, down regulates leptin.

In summary, it is well established that leptin reduces body fat [26, 29, 37], but the mechanism is unknown. No studies have examined whether leptin acts directly on the fat pad to reduce fat mass, or whether it acts indirectly by stimulating another factor. In addition, although it is known that leptin can modify adipocyte metabolism to reduce fat accumulation, it is not clear whether or not leptin also modifies adipocyte growth. The studies in the next chapter will determine whether leptin acts directly or indirectly to reduce fat mass.

Adipogenesis

Adipogenesis is the process by which preadipocytes become mature adipocytes and occurs due to normal cell turnover and increased need for storage of energy excess [94]. Key features of the adipogenesis process include morphological changes, growth

inhibition, lipogenic enzyme expression, lipid accumulation, and hormone sensitivity [94]. When preadipocytes are induced to differentiate, they undergo 2-3 rounds of cell division known as mitotic clonal expansion (MCE) [95].

Soon after MCE begins, preadipocytes begin expressing high levels of CCAAT/enhancer-binding proteins (CEBP) β and δ [95]. After several rounds of MCE, CEBP- β and δ acquire DNA binding activity and transcriptionally activate CEBP- α and peroxisome proliferator-activated receptor γ (PPAR γ) [96-97]. CEBP- α and PPAR γ are antimitotic, so once they are expressed, MCE stops [95-96].

CEBP- α and PPAR γ transcriptionally activate adipocyte marker genes that produce the differentiated adipocyte phenotype [94-97]. These markers include glycerophosphate dehydrogenase (GPDH), fatty acid synthase, acetyl CoA carboxylase, malic enzyme, Glut 4, and aP2 [94, 97]. CEBP- α and PPAR γ are involved in a positive feedback loop with one another, allowing them to remain elevated for the life of the cell and maintain the differentiated state [97].

CEBP- β is essential for MCE because without it, CEBP- α and PPAR γ cannot be activated [98]. Additionally, it has been shown that MCE and PPAR γ , but not CEBP- α , are required for adipogenesis [95,97]. CEBP- α is required for insulin sensitivity and keeping PPAR γ elevated [97].

Angiogenic actions of leptin

Although leptin has been extensively studied for its role in maintaining body weight, other peripheral actions have been observed. Examples include hematopoiesis, angiogenesis, body weight regulation, reproduction, bone metabolism and immune

response [35,99]. Recent studies have shown that there is a strong connection between adipogenesis, angiogenesis and leptin [99-101].

Angiogenesis is the formation of new blood vessels from existing vessels and is essential for providing blood to growing tissues, including adipose tissue [99]. Adipose tissue requires a constant supply of blood to deliver metabolic substrates, such as glucose and lipids, and adipogenic regulators, such as insulin, glucocorticoids, and thyroxine [102]. It is probably for this reason that an inhibition of angiogenesis suppresses adipogenesis [101].

The relationship between leptin, adipogenesis and angiogenesis remains unclear. It is known that leptin receptors are present in endothelial cells [99]. Rupnick et al. [101] showed that mice treated with an angiogenic inhibitor, TNP-470, or leptin both lost a significant amount of weight and decreased proliferation of endothelial cells versus controls. An *in vitro* study examining the effect of leptin on endothelial cell proliferation showed that 10 ng/ml of leptin increased endothelial cell number [99]. The conflicting results could be because there are additional factors *in vivo* that are induced by leptin and decrease endothelial cell proliferation.

Cell Lines vs. Primary Culture

Cell lines cloned from mouse cells have been used for many years to study adipogenesis. The most popular cell lines are a population of homogeneous preadipocytes with aneuploid genomes as opposed to cells from primary culture which are composed of a variety of cell types and have a diploid genome [103-104]. With appropriate differentiation signals, cell lines will differentiate into adipocytes only,

whereas primary cultures can undergo more than one developmental pathway that results in cell types other than adipocytes [104].

Primary cultures of preadipocytes are a better model than cell lines for studying regulation of adipogenesis because it has been shown that fat depots vary with respect to sympathetic innervation, proliferation and differentiation capacity, and hormonal responses [102]. Since cell lines are homogeneous, they do not account for depot specific differences in metabolism. Additionally, primary culture stromal vascular cells and cell lines respond differently to hormones such as insulin, glucocorticoids and growth hormone [105-106].

Overall, cell lines may be helpful when studying terminal differentiation of adipocytes, but primary cultures are ideal when studying what causes cells to commit to the adipocyte lineage [102].

Leptin and Preadipocyte Proliferation and Differentiation

To date, only two studies have examined the effect of leptin on preadipocyte proliferation [67, 69]. Machinal-Quelin et al. [67] exposed stromal vascular cells obtained from male Sprague-Dawley rats to 10 nM (160 ng/ml) murine leptin for 24hrs. Cells showed increased ³H-thymidine incorporation, which was used as a measure of DNA synthesis. Stromal vascular cells were not separated from adipocytes, so it cannot be concluded whether stromal vascular cells, adipocytes, or both were responsible for the increase in proliferation. Ramsay [69] exposed porcine stromal vascular cells to 10, 100, and 1000 ng/ml of porcine leptin or 1000 ng/ml of mouse leptin for 5 days. ³H-thymidine incorporation was also used as a measure of preadipocyte proliferation, and stromal vascular cells were separated from adipocytes [69]. Both the 1000 ng/ml porcine

and mouse leptin doses increased preadipocyte proliferation [68]. The large difference in the dose of leptin required to stimulate proliferation could be due to the different sources of stromal vascular cells and leptin.

Three *in vitro* studies have assessed the effect of leptin on preadipocyte differentiation [67-69]. The first study added 0.6 $\mu\text{g/ml}$ leptin to the human marrow stromal cell line, which contains cells that have the potential to develop into adipocytes or osteoblasts [68]. Cells were exposed to leptin for 48 hours, which decreased adipocyte differentiation as assessed by Oil Red O staining. The second study exposed stromal vascular cells from male Sprague-Dawley rats to 10 nM (160 ng/ml) leptin for 48 hours, resulting in increased adipocyte differentiation as assessed by GPDH activity [67]. The third study exposed porcine stromal vascular cells to 10, 100 and 1000 ng/ml porcine leptin for 5 days and found no difference in preadipocyte differentiation compared with control conditions as measured by the differentiation enzymes GPDH and lipoprotein lipase (LPL) [69].

This project examines whether leptin is able to modify preadipocyte growth. It is possible that leptin directly affects preadipocyte growth by inhibiting/stimulating preadipocyte proliferation and/or differentiation. It is also possible that leptin activates or inhibits another factor responsible for modifying preadipocyte growth. Lastly, it is possible that leptin does not affect preadipocyte growth at all. The experiments in this paper tested the effect of leptin alone, serum from leptin treated animals, and conditioned media from leptin treated animals on preadipocyte proliferation and differentiation in a primary culture of stromal vascular cells obtained from male Sprague-Dawley rats. ^3H -

thymidine incorporation was used as a measure of proliferation, while GPDH activity was used to measure differentiation.

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CHAPTER 3

DIRECT AND INDIRECT EFFECTS OF LEPTIN ON PREADIPOCYTE
DEVELOPMENT

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ABSTRACT

Leptin has been shown to reduce body fat *in vivo*. Adipocytes express the leptin receptor, so it is realistic to expect a direct effect of leptin on adipocyte growth and metabolism. However, *in vitro* studies examining the effect of leptin on adipocyte metabolism require supraphysiologic doses of the protein to see a decrease in lipogenesis or stimulation of lipolysis, implying an indirect action of leptin. It is also possible that leptin reduces adipose mass by inhibiting preadipocyte proliferation (increase in cell number) and/or differentiation (lipid filling). Thus, we determined direct and indirect effects of leptin on preadipocyte proliferation and differentiation *in vitro*. We determined the effect of leptin (0-500 ng/ml), serum from leptin infused rats (0.25% by volume), and adipose conditioned media from leptin infused rats (0-30% by volume) on preadipocyte proliferation and differentiation in a primary culture of cells from male Sprague-Dawley rat adipose tissue. Leptin (50 ng/ml) stimulated proliferation of preadipocytes, but 250 and 500 ng leptin/ml inhibited proliferation of both preadipocyte and stromal vascular fractions, as measured by ³H-thymidine incorporation. Serum from leptin infused rats inhibited proliferation of the adipose ($p < 0.06$) and stromal vascular ($p < 0.05$) fractions, but conditioned media had no effect on proliferation of either cell fraction. None of the treatments changed preadipocyte differentiation as measured by GPDH activity. These results suggest that leptin could inhibit preadipocyte proliferation by modifying release of a factor from tissue other than adipose tissue.

INTRODUCTION

Parabiosis studies in genetically obese mice imply that there is a circulating factor responsible for regulating food intake and body weight [1,2]. Leptin, a circulating hormone produced by the obese (*ob*) gene in adipose tissue [3], has experimentally been shown to reduce body weight by decreasing food intake and increasing energy expenditure in rodents [4-7]. The loss of body weight is specific to fat, as there is no loss in lean mass due to leptin [4]. The exact mechanism by which leptin reduces fat mass is unknown.

Leptin initiates biological responses by binding to its receptor, Ob-R, which is found in the brain and most peripheral tissues, including adipose tissue [8]. Direct injections of leptin into the brain of rodents decrease appetite and body fat [6], so it was initially thought that leptin exerted its effects exclusively by binding receptors in the central nervous system [8]. It has since been shown that peripheral leptin infusions in mice reduce body weight and food intake [9]. Additionally, peripheral infusions of leptin have been shown to decrease body weight without affecting food intake, implying that leptin may act directly on leptin receptors in peripheral tissues to reduce body fat [10].

In addition to reducing the size of fat depots, leptin acts on many other peripheral tissues and is involved in several other physiological processes, such as hematopoiesis, angiogenesis, lipid metabolism, reproduction, bone metabolism and immune response [11,12]. In adipose tissue, leptin stimulates lipolysis *in vitro* [13-15] and *in vivo* [16] and inhibits lipogenesis *in vitro* [17-18]. The *in vitro* studies indicate that leptin has the potential to directly activate an adipose tissue receptor. *In vivo*, however, it is unclear whether leptin stimulates lipolysis in adipocytes directly or through activation of the

sympathetic nervous system since leptin activates sympathetic outflow to brown adipose tissue and possibly white adipose tissue [18-20].

In addition to increasing lipolysis and decreasing lipogenesis, leptin may affect the size of adipose depots by increasing or decreasing preadipocyte proliferation and/or differentiation. Since adipose tissue contains leptin receptors [21], it is possible that leptin directly influences preadipocyte growth. Alternatively, leptin could indirectly affect preadipocyte growth by activating or inhibiting another factor. *In vitro* studies examining leptin's effect on lipolysis require very large concentrations of leptin to see an increase in lipolysis [13-15], while *in vivo* studies require much lower concentrations [16]. This implies that leptin may not act directly to inhibit lipolysis. It cannot be ruled out that leptin acts through the sympathetic nervous system to regulate adipocyte metabolism and growth.

Only two studies have examined the effect of leptin on preadipocyte proliferation [22, 23]. One study showed that 160 ng/ml murine leptin increased preadipocyte proliferation in a primary culture of rat stromal vascular cells, but stromal vascular cells were not separated from adipocytes, so it is uncertain whether the cell type that increased was preadipocytes, stromal vascular cells or both [22]. The other study showed increased proliferation in porcine preadipocytes treated with 1,000 ng/ml porcine leptin [23]. The large difference in the dose required to increase proliferation could be due to the different sources of preadipocytes and leptin. Three studies have examined the effect of leptin on preadipocyte differentiation *in vitro* [22-24]. One study used preadipocytes from a human marrow stromal cell line, which resulted in decreased differentiation due to leptin [24]. The second study used stromal vascular cells from white fat obtained from male

Sprague-Dawley rats, resulting in increased differentiation [22]. The last study found no effect of 10, 100 or 1,000 ng/ml porcine leptin on differentiation of pig preadipocytes [23]. The different sources of preadipocytes could account for the variability in results.

The purpose of the studies described here was to determine if leptin affects proliferation and/or differentiation of preadipocytes derived from white fat stromal vascular cells, and, if there is an effect, whether it is due to a direct action of leptin on the cells.

MATERIALS AND METHODS

Cell Culture Techniques

Inguinal fat pads were removed aseptically from male Sprague-Dawley rats (Harlan Industries, Indianapolis, IN), weighing 80-100 g, following the technique described by Marques et al [25]. Briefly, rats were anesthetized with ketamine (200mg/kg body weight) and xylazine (10mg/kg) before removal of inguinal fat pads in sterile conditions. Fat was then minced and incubated with digestion buffer (8 ml HEPES and 1000 units/ml collagenase) for 90-120 minutes at 37° C. The fat was filtered through 240 and 20 µm mesh filters, and undigested fat was discarded. Digested cells were resuspended in Dulbecco's Modified Eagle's Medium (DMEM)/F12 Ham's Medium (Sigma, St. Louis, Missouri) and centrifuged at 2000 rpm for 10 minutes to separate mature adipocytes from the stromal vascular (SV) cell pellet. Floating fat filled adipocytes were discarded. Purgene Red Blood Cell Lysis Solution (Gentra Systems, Inc., Minneapolis, MN) was added to SV cells for 5 minutes, after which SV cells were resuspended in DMEM/F12 + 10% Fetal Bovine Serum (FBS) and centrifuged at 1800

rpm for 5 minutes. A small aliquot of SV cells was diluted with Rappaport's stain and counted on a hemocytometer.

Proliferation Assay

On *Day 0*, cells were plated at a density of 4.8×10^3 cells/cm² on 12.5 cm² tissue culture flasks in 2 ml of plating media (DMEM/F12 medium, 50 mg/L gentamycin sulfate, 50 mg/L cefazolin, 2.5 mg/L amphotericin, 10% fetal bovine serum). Cells were incubated with plating media at 37 ° C and 5 % humidity for 24 hours, followed by incubation in serum free media for 24 hours. Treatment media was added to the assay on *Day 2*, as noted below. On *Day 4* treatment media was replaced with fresh treatment media containing ³H-thymidine (0.5μCi/flask). Twenty-four hours later, ³H-thymidine was removed with cold thymidine rinse media (12 mg thymidine/1000ml DMEM). Cells were placed on lipid filling media (DMEM/F12, 10% Pig Serum, 1.0nM insulin, 10units/ml heparin), which was changed every other day until *Day 13*. On *Day 14*, cells were enzymatically harvested using Hank's Balanced Salt Solution (HBSS: Sigma H-1387) containing 0.5% bovine serum albumin, 0.5 mg/ml trypsin, and 125 units/ml collagenase. Lipid filled adipocytes were separated from stromal vascular cells by density gradient centrifugation using a Percoll gradient, as described by Novakofski [26]. Each cell fraction was transferred to a scintillation vial with scintillation cocktail. The incorporation of [³H] thymidine into stromal vascular cells or adipocytes was measured using scintillation counting. Data is expressed as pmol of thymidine incorporated per flask.

Differentiation Assay

On *Day 0*, cells were plated on 35 mm dishes at a density of 1×10^4 cells/cm² in 2 ml of plating media (same as in proliferation assay above). Cells were incubated with plating

media at 37° C and 5 % humidity for 24 hours, followed by incubation in serum free media for 24 hours. On *Day 2*, serum free treatment media was added to cells as described below. Fresh treatment media was added to cells on *Days 4* and *6*. On *Day 8*, cells were harvested in sucrose buffer (0.25 M, pH 7) and frozen at -80° C until assayed for *sn*-glycerophosphate dehydrogenase (GPDH) activity as described by Wise and Green [26] and as modified by Ramsay et al [28]. Protein was measured using the Bio-Rad assay (Bio-Rad, Melville, New York). Results are expressed as mUnits/min/mg protein (1U is equal to the oxidation of 1nmol NADH).

Conditioned Media and Serum from Leptin Treated Rats

Fourteen male Sprague-Dawley rats (Harlan Industries) weighing between 320 and 340 grams were housed in hanging wire mesh cages at 23° C, with lights on 12 hours/day starting at 7:00 a.m., for 1 week. Rats were fed a standard diet of Purina Rodent chow 5001 (Purina Mills, St. Louis, MO). After a 7 day acclimation period, daily food intake and body weight were recorded until the rats were sacrificed. After 3 days of baseline measures, rats were weight matched into two groups and fitted with i.p. Alzet miniosmotic pumps (Direct Corp. Cupertino, CA Model 1007D) which delivered either 100 µg recombinant rat leptin (R&D Systems, Minneapolis, MN) or an equivalent volume of PBS per 24 hours for five days. Blood was collected on the third day of infusion by tailbleed for measurement of serum leptin (Rat Leptin RIA kit: Linco, St. Louis, MO). After 5 days of infusion, rats were killed, trunk blood collected, and epididymal, retroperitoneal, perirenal, and mesenteric fat pads dissected and weighed. Serum leptin concentration was measured and serum was added to the cell culture assays where indicated below.

Epididymal and retroperitoneal fat pads from the leptin and PBS infused rats were washed with saline and weighed. A small portion (50-100 mg) of fat was fixed in osmium tetroxide solution for determination of cell number by Coulter Counter and Multisizer as described previously [25]. Large blood vessels were removed from the remaining fat, which was minced and washed 3-4 times with 5-10 volumes of Hank's Balanced Salt Solution (HBSS) and filtered through Fisherbrand P8 filter paper (Fisher Scientific, Pittsburgh, PA) to remove excess solution. Fat was reweighed and incubated with 10 volumes of DMEM/F12 in 12.5 cm³ incubation flasks for 4 hours at 37° C and 5% humidity. DMEM/F12 containing no adipose tissue was incubated and used as a control in the cell culture assays, as noted below. Following the incubation period, fat and DMEM/F12 were filtered through Fisherbrand P8 filter paper. The filtered media was then sterile filtered (Steriflip Filters, .22 µm,) and frozen at -80° C and later diluted with varying amounts of DMEM/F12 to equalize volume based on cell number counts.

Experiment 1

Cells were cultured as described above. Proliferation and differentiation assays were performed on 6 different pools (1 pool = 2 rats) of cells and treatments were run in quadruplicate on each pool of cells. Treatment media for the proliferation assay consisted of 2 ml of DMEM/F12 plus 0.25% by volume control rat serum and varying concentrations of leptin (0, 5, 50, 100, 250, 500 ng/ml). The control rat serum consisted of pooled rat serum collected from rats in previous studies, and it served as an internal control as it was used in all of the proliferation assays. AraC (536 nM), a DNA synthesis inhibitor, was used as a negative control for preadipocyte proliferation, while 10 nM IGF-1 was used as a

positive control. Data is expressed as a percent of control, which is the 0 ng/ml leptin treatment.

Treatment media for the differentiation assay consisted of serum free DMEM/F12, 10 nM insulin, 60 nM transferrin, 2 nM thyroxine and varying concentrations of leptin (0, 5, 50, 100, 250, 500 ng/ml). TNF- α (1 nM) was used as a negative control for preadipocyte differentiation, and 1000 nM insulin was used as a positive control. Data is expressed as percent of control, which is the 0 ng/ml concentration of leptin.

LDH Assay

On days 4 and 5 of the proliferation assay, media from cells treated with 100 ng/ml leptin and AraC was collected, using an LDH kit (Sigma DG1340-K) following manufacturer protocol, to test for LDH release into the media, which is a measure of cell damage.

Experiment 2

Cells were cultured and proliferation and differentiation assays were performed as described above. Treatment media consisted of 2 ml DMEM/F12 containing 0.25% serum by volume from each of the leptin and PBS infused rats (n=7) or various concentrations by volume of conditioned media (5, 10, 20, or 30% by volume). Each conditioned media treatment was diluted to be equivalent of having been exposed to 150,000 adipocytes/ml. Serum and conditioned media collected from the same rat were added as treatments to the same pool of cultured cells. Positive and negative controls were the same as in Experiment 1. Conditioned media that had not been exposed to adipose tissue (5, 10, 20, or 30%), as described above, was also used as a control. Additionally, DMEM/F12 and control rat serum (explained in experiment 1) served as a control for the proliferation assay, while

DMEM/F12, 10 nM insulin, 60 nM transferring and 2 nM thyroxine served as a control for the differentiation assay. All data was taken as a percent of these controls. Conditioned media was analyzed for fatty acids (NEFA C Kit, Wako Chemicals, GmbH, Germany) and triglycerides (L-Type TG-H Kit, Wako Chemicals) following the manufacturer's protocol.

Statistics

One-way ANOVA (Statistica Software 99' Edition, Stat Soft, Tulsa, OK) was used to compare dose dependent differences in preadipocyte and stromal vascular proliferation and preadipocyte differentiation in Experiment 1. Two-way ANOVA was used to compare the effects of type and volume of media on preadipocyte and stromal vascular proliferation or preadipocyte differentiation in Experiment 2. Post hoc comparisons were made using Duncan's multiple-range test. An unpaired t-test was used to compare the differences in body weight, fat pad weight, food intake and serum leptin levels of the infused rats and to test the effect of serum from leptin or PBS infused rats on preadipocyte and stromal vascular proliferation in Experiment 2. Differences were considered significant at $p < 0.05$.

RESULTS

Experiment 1

Leptin had a biphasic effect on preadipocyte proliferation. A concentration of 50 ng/ml leptin significantly increased preadipocyte proliferation ($p < 0.05$), while 250 and 500 ng/ml leptin significantly decreased preadipocyte proliferation ($p < 0.01$: **Figure 1**). Likewise, 250 and 500 ng/ml leptin significantly decreased stromal vascular cell proliferation ($p < .01$: **Figure 2**). As expected, IGF-1 stimulated proliferation of the adipose and stromal vascular fractions, as compared to controls, by 49 and 23%,

respectively (**Figures 1 and 2**). AraC decreased preadipocyte and stromal vascular cell proliferation compared with controls by 45 and 83% respectively (**Figures 1 and 2**).

Leptin had no effect of preadipocyte differentiation as measured by GPDH activity (**Figure 3**). As expected, TNF- α decreased GPDH activity by 59%, while 1000 nM insulin increased GPDH activity by 59% compared with controls (**Figure 3**).

Media collected from 100 ng/ml leptin or AraC treatments had significantly less LDH activity than the lipid calibrator provided by the manufacturer (**Figure 4**), indicating that the inhibition of proliferation in cells treated with leptin or AraC was not associated with cell death.

Experiment 2

There was no difference in beginning body weight, end body weight, food intake or fat pad weights of rats infused with leptin or PBS for 5 days of leptin infusion (**Table 1**). Leptin infused rats gained significantly less weight during infusion and had significantly higher serum leptin levels on both days 3 and 5 of infusion compared with PBS infused rats ($p < 0.05$; **Table 1**). The control rat serum contained significantly more leptin (8.3 ng/ml) than serum from leptin or PBS infused rats on day 5.

Serum from leptin treated rats inhibited proliferation of preadipocytes ($p = 0.058$) and of stromal vascular cells ($p < 0.05$) compared to serum from PBS infused rats, but the inhibition was only significant in the stromal vascular fraction (**Figure 5**). Preadipocyte differentiation was not affected by serum from leptin or PBS infused rats (**Figure 6**).

There was no difference in preadipocyte or stromal vascular cell proliferation or on preadipocyte differentiation due to adipose tissue conditioned media from leptin or PBS infused rats, and there was no interaction between volume and source of media on

proliferation (conditioned or control) (**Figures 7-9**). There was a treatment effect of adipose tissue conditioned media compared with control conditioned media that had not been exposed to adipose tissue, as all levels of adipose conditioned media significantly increased preadipocyte differentiation compared with conditioned media not exposed to adipose tissue (**Figure 9**).

Conditioned media from leptin and PBS infused rats contained low levels of fatty acids and triglycerides (**Table 2**). IGF-1 and AraC significantly increased and decreased preadipocyte and stromal vascular proliferation, respectively ($p < 0.05$: **Figures 7 and 8**). Preadipocyte differentiation was significantly increased by 1000 nM insulin and decreased by 1 nM TNF- α (**Figure 9**).

DISCUSSION

In this experiment, we examined whether leptin modifies preadipocyte development (proliferation and/or differentiation) directly and/or indirectly. Leptin alone had a biphasic effect on preadipocyte proliferation. A relatively low dose of 50 ng/ml leptin significantly increased preadipocyte proliferation, while 250 and 500 ng/ml leptin significantly inhibited both preadipocyte and stromal vascular proliferation ($p < 0.05$). In contrast, relatively low concentrations of serum from leptin infused rats decreased proliferation of adipose and stromal vascular cell fractions compared with serum from PBS infused rats, although the inhibition did not quite reach significance in the adipose fraction ($p < 0.06$). This implies that leptin can modify the rate of growth of adipose depots indirectly, possibly by activating or inhibiting another factor, to decrease proliferation. Although serum from leptin infused rats decreased preadipocyte and stromal vascular proliferation, adipose tissue conditioned media had no effect on

preadipocyte or stromal vascular proliferation, indicating that the factor responsible for decreasing proliferation is not released from fat. Neither leptin nor serum from leptin-infused rats influenced preadipocyte differentiation. Adipose conditioned media from both leptin and PBS infused rats significantly increased preadipocyte differentiation, implying that there is a factor released from fat that stimulates preadipocyte differentiation.

It may not be surprising that leptin increased preadipocyte proliferation since the protein increases proliferation of other cell types, such as hematopoietic and endothelial cells [11]. Additionally, other investigators have reported that leptin increases preadipocyte proliferation in primary culture [22-23]. The results from this study demonstrate that leptin increases proliferation of both preadipocytes and stromal vascular cells and is not specific to either cell type.

Adipocytes express the leptin receptor [21], so it is reasonable to expect a direct effect of leptin on adipocyte growth and metabolism. The amount of leptin required to stimulate proliferation in preadipocytes *in vitro* was higher than the circulating concentrations found in normal animals. In young rats, physiologic levels of circulating leptin are about 5 ng/ml and levels increase with age and adiposity [29]. The amount of leptin surrounding the adipocyte is not known, but since leptin is secreted from adipocytes, there is probably a lapse in time between the protein's secretion and entry into circulation, allowing leptin to accumulate in the interstitial space. Additionally, expression of leptin mRNA varies across different fat depots and correlates with fat cell size [11, 30-33]. Fat pads have varying capacities to proliferate and differentiate [34], and tend to proliferate after existing adipocytes fill with lipid [30]. Therefore, it is

possible that 50 ng/ml leptin is in the physiological range for leptin levels near the adipocyte in some fat depots. The media used in the proliferation assay provided an abundance of nutrients, and contained high concentrations of insulin, which are similar to conditions seen in obesity *in vivo*. It is therefore possible that the 50 ng/ml leptin concentration provided optimal proliferation conditions when coupled with the insulin and nutrients available in the media.

Leptin significantly decreased proliferation of the preadipocyte and stromal vascular cell fractions at higher doses (250 and 500 ng/ml). These leptin concentrations could be so high that leptin nonspecifically bound to other cytokine receptors to inhibit proliferation, especially since the inhibition was not specific to preadipocytes. Other cytokines that possibly inhibit preadipocyte proliferation are IL-4 [35], IL-11 [36], and IL-15 [37]. Alternatively, leptin at high doses could interfere with insulin signaling [38-39]. In primary rat adipocytes, >30 nM (480 ng/ml) leptin almost completely inhibits metabolic activities associated with insulin, such as stimulation of glucose transport, glycogen synthase, lipogenesis, and protein synthesis [39]. Insulin is a mitogenic factor, and has been shown to increase proliferation and differentiation of preadipocytes in cell lines [40-41]. Leptin and insulin activate many of the same pathways, such as mitogen activated protein kinase (MAPK), signal transducer and activator of transcription 1 and 3 (STAT1 and STAT3), and phosphoinositol-3 kinase (PIK3) [38], therefore, high concentrations of leptin could activate these pathways and occupy downstream proteins, leaving no proteins for insulin signaling. A final possibility for the inhibition of proliferation would be a reduction in the number of viable cells present. Central and peripheral administration of leptin has been shown to increase apoptosis of adipocytes

[42-43], so it cannot be ruled out that higher doses of leptin caused cell death, however, in this study necrosis was not present in cells treated with 100 ng/ml leptin.

Leptin had no direct effect on preadipocyte differentiation, consistent with the results of Ramsay [23]. Serum and adipose tissue conditioned media from leptin infused rats did not affect preadipocyte differentiation compared to serum or adipose tissue conditioned media from PBS infused rats. As expected, positive and negative controls increased and decreased preadipocyte differentiation respectively, so if leptin alone, serum from leptin infused animals, or adipose tissue conditioned media from leptin infused animals induced a change in preadipocyte differentiation, the effect would have been seen. It is surprising that leptin does not affect preadipocyte differentiation directly or indirectly since fatty acid synthesis [15,17] and the size of lipid stores are reduced in mice that are infused with leptin [9]. We used a primary culture of stromal vascular cells, whereas studies examining leptin's effect on lipogenesis used differentiated adipocytes from preadipocyte cell lines [15] and primary culture [17]. Therefore, the difference in maturity of preadipocytes compared with differentiated adipocytes could explain why we did not see an effect of leptin on preadipocyte differentiation.

As expected, leptin infused rats in experiment 2 had significantly higher serum leptin levels than PBS infused controls on day 5 of leptin infusion. There was no difference in beginning or end body weight, fat pad weights, or total food intake between leptin and PBS infused rats. Additionally, the control rat serum used in experiment 1 to test the direct effect of leptin on preadipocyte proliferation and differentiation contained a significantly higher amount of leptin than serum from both leptin and PBS infused rats on day 5 of infusion. Therefore, decreased food intake and high serum leptin concentrations

do not account for the decrease in preadipocyte and SV proliferation in cells treated with serum from leptin infused rats.

SNS innervation in WAT decreases fat cell number [44]. Leptin has been shown to activate SNS outflow to brown adipose tissue and to the renal nerves [18], but it is unclear whether leptin stimulates SNS outflow to WAT [45]. Since the serum from leptin treated rats did not contain nerves, SNS nerve activity in adipose tissue was not responsible for inhibiting preadipocyte and stromal vascular proliferation in cells treated with the serum. It is possible, however, that leptin stimulated the SNS to release a factor, such as norepinephrine (NE), which has been shown to inhibit preadipocyte proliferation [46]. This is not likely the factor responsible for decreased proliferation in this study, as NE becomes unstable once blood is collected unless preservatives are added [47].

It is possible that leptin acts indirectly to reduce proliferation by activating or inhibiting another factor since serum from leptin treated animals, but not leptin alone, decreased proliferation of preadipocytes and stromal vascular cells. It would not be surprising that leptin acts indirectly to affect adipocyte growth since it takes much higher leptin concentrations to increase in lipolysis *in vitro* compared to *in vivo* [13,16]. Additionally, leptin increases endothelial cell proliferation *in vitro*, but decreases proliferation *in vivo*, implying that there are additional factors *in vivo* required for leptin to decrease endothelial cell proliferation [48-49].

The source of the serum factor responsible for decreasing proliferation is unknown. Leptin is produced by WAT in proportion to the amount of fat present [33] and regulates the size of WAT [4], so it is reasonable to assume that the factor might be produced in adipose tissue, in proportion to fat depot size. If leptin activates a factor that is released

from adipose tissue to inhibit preadipocyte and stromal vascular proliferation, adipose tissue conditioned media from leptin infused rats should inhibit proliferation of these cell types as well. We found no difference in preadipocyte or stromal vascular proliferation in cells treated with conditioned media from leptin or PBS infused rats or non-adipose control conditioned media at any dose tested, indicating that leptin does not activate a factor released from fat to decrease proliferation. In addition to adipose tissue, the leptin receptor is found in various tissues, such as the hypothalamus, liver, pancreatic β -cells, and muscle, and leptin has been shown to exert direct effects on these tissues [50]. It is possible that leptin acts directly on one of these tissues to stimulate the release of another factor that is responsible for decreasing preadipocyte proliferation.

Many studies have examined the relationship between leptin and other cytokines, hormones, and factors secreted from non-adipose tissues [37,51-57]. IL-15 is a cytokine involved in T-cell stimulation, enhancement of natural killer cells, muscle protein accretion, and possibly direct reduction of adipose mass and is expressed mainly in skeletal muscle [37,51]. IL-15 and its receptor are expressed in adipose tissue, and subcutaneous administration of IL-15 results in decreased fat mass without an inhibition of food intake or a decrease in muscle mass [37,51]. These metabolic responses to IL-15 administration are very similar to those caused by leptin administration. *In vitro*, IL-15 decreases lipolysis by activating the Protein Kinase A and Jak-Stat pathways, which are the two pathways that leptin activates [51]. Leptin has been shown to increase IL-15 activation of NK cells *in vitro* [52] and, *in vivo*, IL-15 administration decreases circulating leptin levels [53]. Therefore, it is possible that leptin activates IL-15 to inhibit

preadipocyte proliferation, and, in turn, IL-15 downregulates leptin. Future studies should examine the relation between circulating concentrations of leptin and IL-15.

α -melanocyte stimulating hormone (α -MSH) is derived from the precursor protein produced by the pro-opiomelanocortin (POMC) [54]. The POMC gene is highly expressed in the arcuate nucleus (ARC) and other parts of the hypothalamus, as well as in a variety of peripheral tissues at lower levels [55]. α -MSH acts on melanocortin receptor subtypes MC3-R and MC4-R to inhibit food intake and increase energy expenditure [54-55]. POMC neurons in ARC express the leptin receptor and adipose tissue contains the MC4 receptor [55-56]. Like leptin, α -MSH acts in the periphery, as well as the hypothalamus, to reduce body weight [54-55]. Activation of melanocortin receptors by a synthetic analog of α -MSH in WAT inhibits agouti stimulation of PPAR γ in adipose tissue, implying that stimulation of these receptors has a direct effect on adipose tissue [58]. Peripheral injections of an α -MSH analog into ob/ob mice result in significant weight loss [41]. Recent studies have shown that leptin and α -MSH control each other's expression and secretion [55,57]. Circulating α -MSH levels are lower in ob/ob mice lacking leptin than in lean controls, and ip administration of leptin to these mice doubles circulating α -MSH [57]. *In vitro*, α -MSH has been shown to decrease leptin expression and secretion [55]. Therefore, a possible feedback loop exists between α -MSH and leptin, in which leptin increases α -MSH secretion and α -MSH downregulates leptin expression and secretion.

Adipose tissue conditioned media from both leptin and PBS infused rats significantly increased preadipocyte differentiation versus control conditioned media, implying that something released from fat promotes lipid filling. Fatty acids and

triglycerides were present at very low levels in conditioned media, therefore they do not account for the increase in preadipocyte differentiation. Other factors released from fat, such as IGF-1, could be responsible for increased preadipocyte differentiation [30], as IGF-1 has been shown to increase preadipocyte differentiation in cell lines [41].

Further studies are needed to determine which factor leptin activates and/or inhibits to decrease preadipocyte and stromal vascular proliferation. *In vitro* studies testing the effect of factors like α -MSH or IL-15 alone, and in the presence of leptin, on preadipocyte and stromal vascular proliferation would lead to a better understanding of leptin's role in preadipocyte and stromal vascular proliferation. Additionally, the factor responsible for decreasing preadipocyte and stromal vascular proliferation could be a novel factor. In this case, proteomics could be used to identify the factor.

In summary, leptin has been shown to affect adipocyte metabolism [13-17], but it is unclear whether the effect is direct or indirect. In our studies, we show that leptin acts directly on adipose tissue to increase preadipocyte proliferation and indirectly to inhibit preadipocyte and SV proliferation. The inhibition could be due to the activation or inhibition of a factor, and the factor could be a novel factor. Although leptin has direct and indirect effects on proliferation, it does not affect preadipocyte differentiation. The results of these studies reinforce the complexity of leptin's actions, but provide important insights into its role in preadipocyte development. Discovery of the factor that leptin activates and/or inhibits to decrease preadipocyte proliferation will lead to a better understanding of fat cell development, and hopefully contribute to the search for effective treatment of obesity.

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Table 1: Body weight, change in body weight, fat pad weights and leptin levels after leptin and PBS infusion for 5 days.

	Leptin	PBS
Beginning Body Weight (g)	347 ± 3	349 ± 4
End Body Weight (g)	342 ± 5	351 ± 5
Change in Body Weight (g/5 day)	-4.8 ± 3.2 *	2.3 ± 2.2
Total food intake (g)	89 ± 6	95 ± 2
Epididymal Fat (g)	3.2 ± 0.22	2.7 ± 0.15
Retroperitoneal Fat (g)	0.83 ± 0.12	1.0 ± 0.13
Perirenal Fat (g)	0.50 ± 0.06	0.55 ± 0.05
Mesenteric Fat (g)	1.2 ± 0.19	1.5 ± 0.17
Serum leptin Day 3 (ng/ml)	5.3 ± 1.3 *	1.7 ± 0.17
Serum leptin Day 5 (ng/ml)	3.9 ± 0.78 *	1.73 ± 0.21

Values are means ± sem for 6 rats in the leptin group and 7 in the PBS group.

* indicates a significant difference ($p < 0.05$) between leptin and PBS infused rats.

Table 2: Triglyceride and fatty acid levels in adipose tissue conditioned media from leptin and PBS infused rats.

	Leptin	PBS
Fatty Acids (ng/ml)	0.09 ± 0.01	0.10 ± 0.01
Triglycerides (mEq/L)	31 ± 7	32 ± 5

Values are means $\pm$ sem for 6 rats in the leptin group and 7 in the PBS group.

There are no significant differences between fatty acid and triglyceride levels in conditioned media from leptin or PBS infused rats.

FIGURE LEGEND

Figure 1: Effect of increasing concentrations of leptin on preadipocyte proliferation, as measured by ^3H -thymidine incorporation. Data are means $\pm$ sem for 3 to 6 replicate cultures (1 replicate = 1 digest = 2 rats), with each treatment determined in quadruplicate. Superscripts denote significantly different preadipocyte proliferation values within the leptin dose response, $p < 0.05$. Leptin significantly increased preadipocyte proliferation from control levels at the 50 ng/ml level. At higher leptin levels, 250 and 500 ng/ml, preadipocyte proliferation was significantly lower than control levels. An asterisk denotes significantly different rates of preadipocyte proliferation between positive (10 nM IGF-1) and negative (536 nM AraC) controls compared with the 0 ng/ml leptin control ($p < 0.05$).

Figure 2: Effect of increasing concentrations of leptin on stromal vascular proliferation, as measured by ^3H -thymidine incorporation. Data are means $\pm$ sem for 3 to 6 replicate cultures (1 replicate = 1 digest = 2 rats), with each treatment determined in quadruplicate. Superscripts denote significantly different preadipocyte proliferation values within the leptin dose response at $p < 0.05$. Stromal vascular (SV) cell proliferation was inhibited by high doses of leptin (250 and 500 ng/ml). An asterisk denotes significantly different rates of SV proliferation between positive (10 nM IGF-1) and negative (536 nM AraC) controls compared with the 0 ng/ml leptin control.

Figure 3: Effect of increasing concentrations of leptin on preadipocyte differentiation, as measured by GPDH activity. Data are means $\pm$ sem for 3 to 6 replicate cultures or digests (1 digest = 2 rats), with each treatment added to dishes in quadruplicate. There was no significant difference in preadipocyte differentiation between any of the leptin doses tested. Insulin and TNF- α served as positive and negative controls, respectively. An asterisk represents a significant difference in differentiation compared to the 0 ng/ml leptin control, $p < 0.05$.

Figure 4: Cell viability as measured by LDH activity. Data are means $\pm$ sem for 4 to 6 replicates, each determined in quadruplicate. An asterisk represents significantly lower LDH activity in media collected from cells treated with 100 ng/ml leptin and AraC for 1 and 2 days compared with LDH assay control, $p < 0.05$.

Figure 5: Effect of serum from leptin and PBS infused rats ($n = 6-7$) on preadipocyte and stromal vascular proliferation. Data are means $\pm$ sem, with each treatment determined in triplicate. Serum from leptin infused rats decreased preadipocyte proliferation vs. serum from PBS infused rats, but the difference did not quite reach significance, $p = 0.058$. Serum from leptin infused rats significantly reduced SV proliferation compared to serum from PBS infused rats, $p < 0.05$.

Figure 6: Effect of serum from leptin and PBS infused rats ($n = 6-7$) on preadipocyte differentiation. Data are means $\pm$ sem, with each treatment determined in triplicate.

There was no difference in preadipocyte differentiation due to serum from leptin or PBS infused rats.

Figure 7: Effect of increasing concentrations of adipose tissue conditioned media ($n= 6-7$) on preadipocyte proliferation. Data are mean $\pm$ sem, with each treatment determined in triplicate. There was no difference in preadipocyte proliferation within or between leptin and PBS infused groups. There was no interaction between volume of media or type of media. An asterisk represents significantly different rates of preadipocyte proliferation in positive (IGF-1) and negative (AraC) controls compared with control conditions, $p < 0.05$.

Figure 8: Effect of increasing concentrations of adipose tissue conditioned media ($n= 6-7$) on SV proliferation. Data are means $\pm$ sem, with each treatment determined in triplicate. There was no difference in SV proliferation within or between leptin and PBS infused groups. There was no interaction between volume or type of media. An asterisk represents significantly different rates of SV proliferation in positive (IGF-1) and negative (AraC) controls compared with control conditions, $p < 0.05$.

Figure 9: Effect of increasing concentrations of adipose tissue conditioned media ($n= 6-7$) on preadipocyte differentiation. Data are means $\pm$ sem, with each treatment determined in triplicate. There was no difference in differentiation within or between leptin and PBS infused groups. An asterisk indicates that there was an effect of adipose tissue conditioned media versus control media not exposed to adipose tissue, $p < 0.05$. A

indicates a significant difference in preadipocyte differentiation rates compared with control conditions, $p < 0.05$.

Figure 1

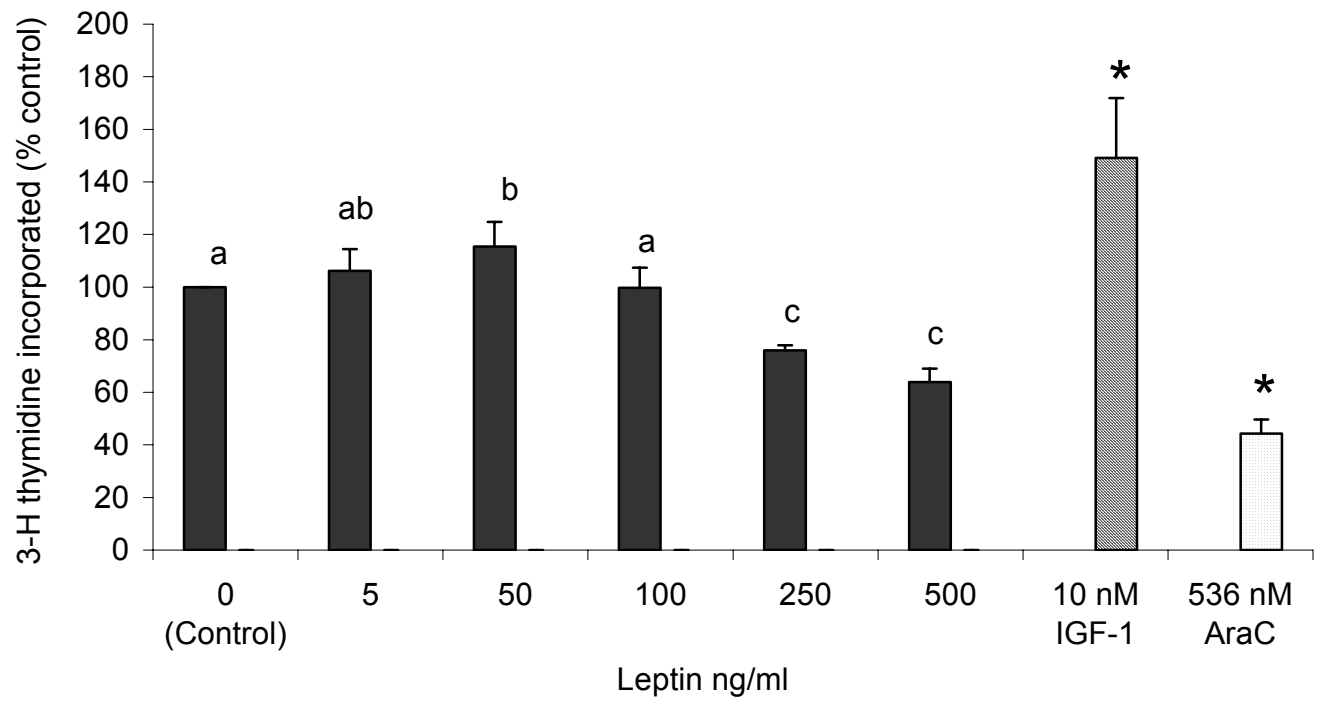


Figure 2

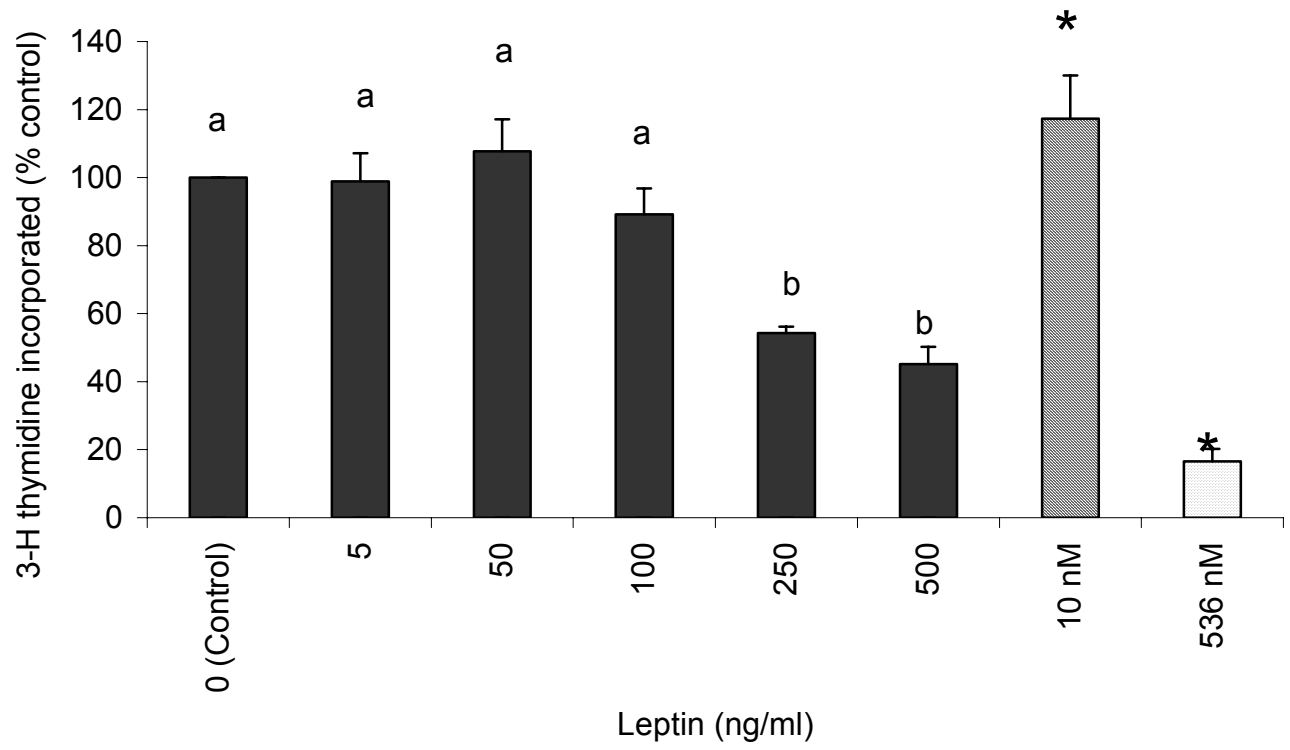


Figure 3

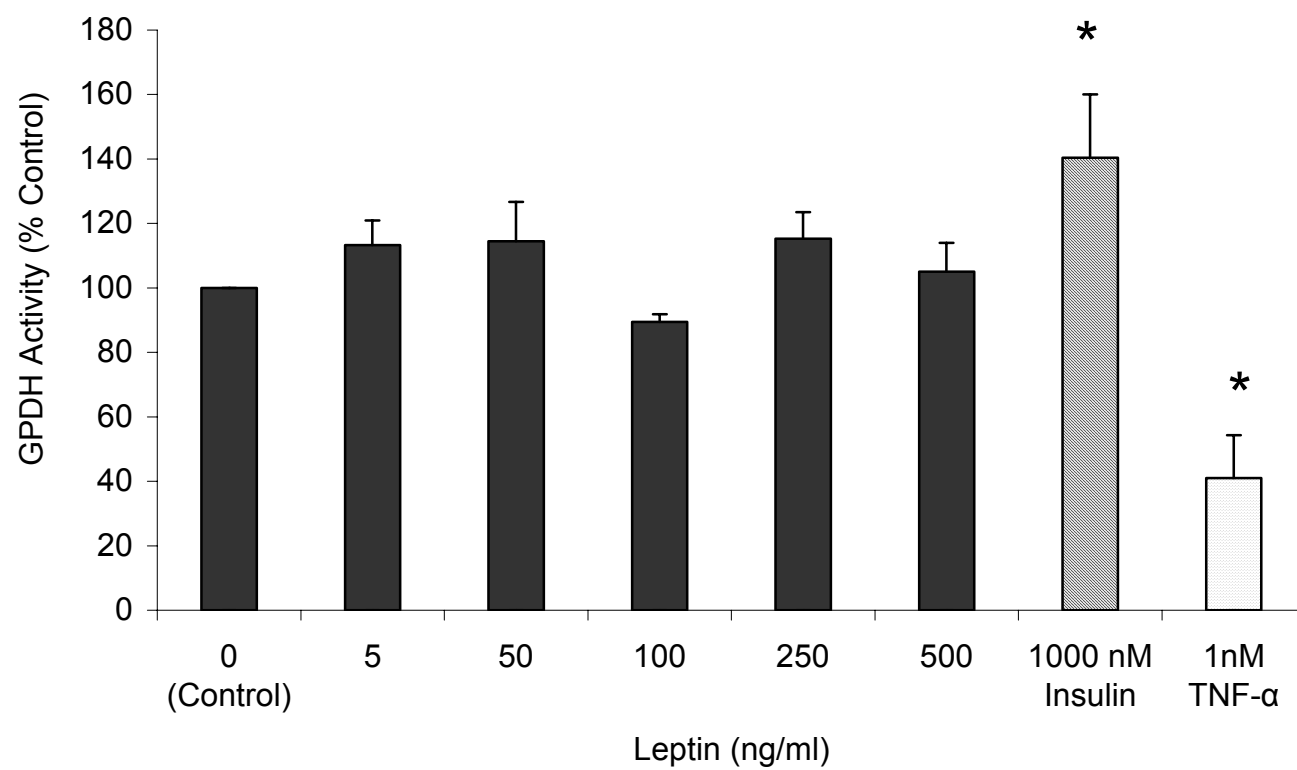


Figure 4

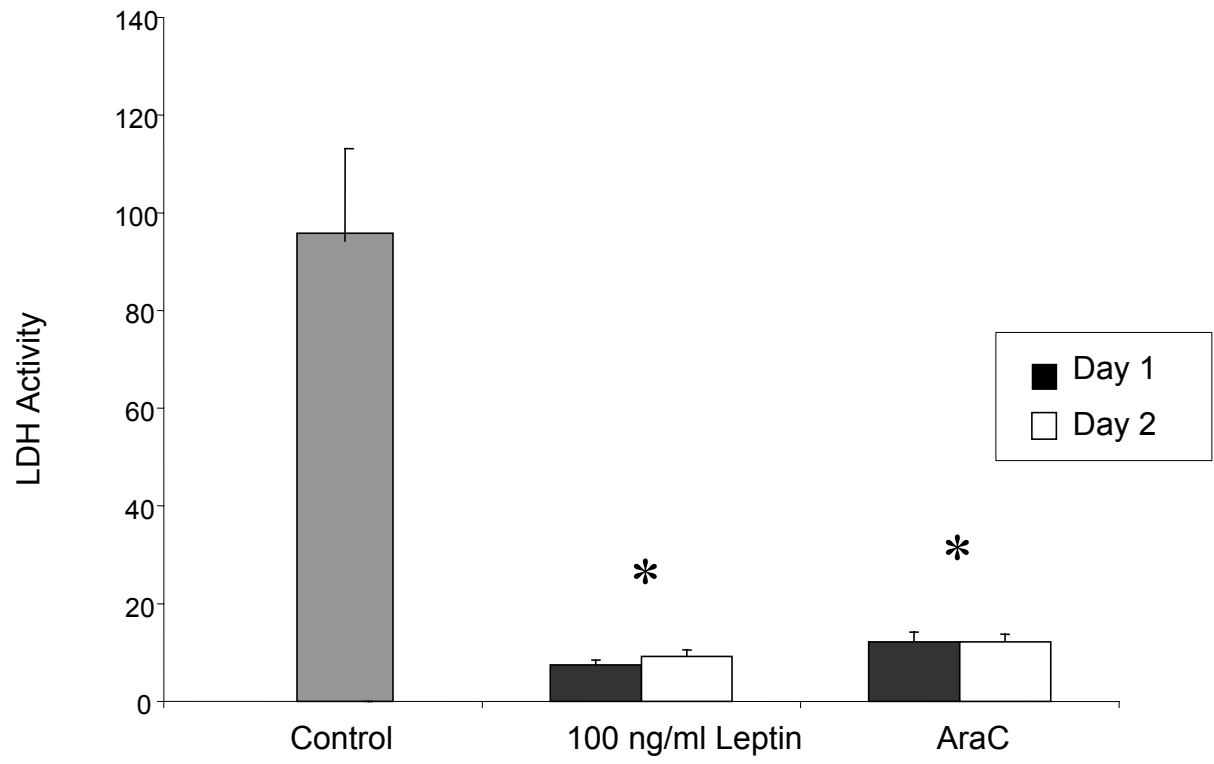


Figure 5

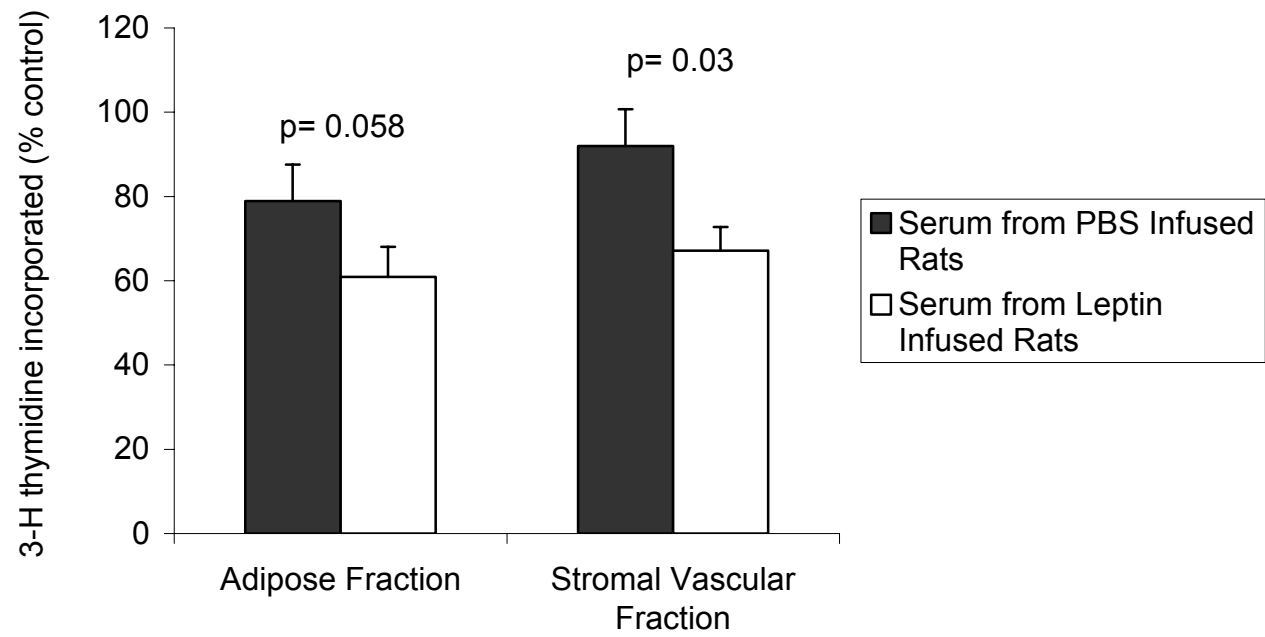


Figure 6

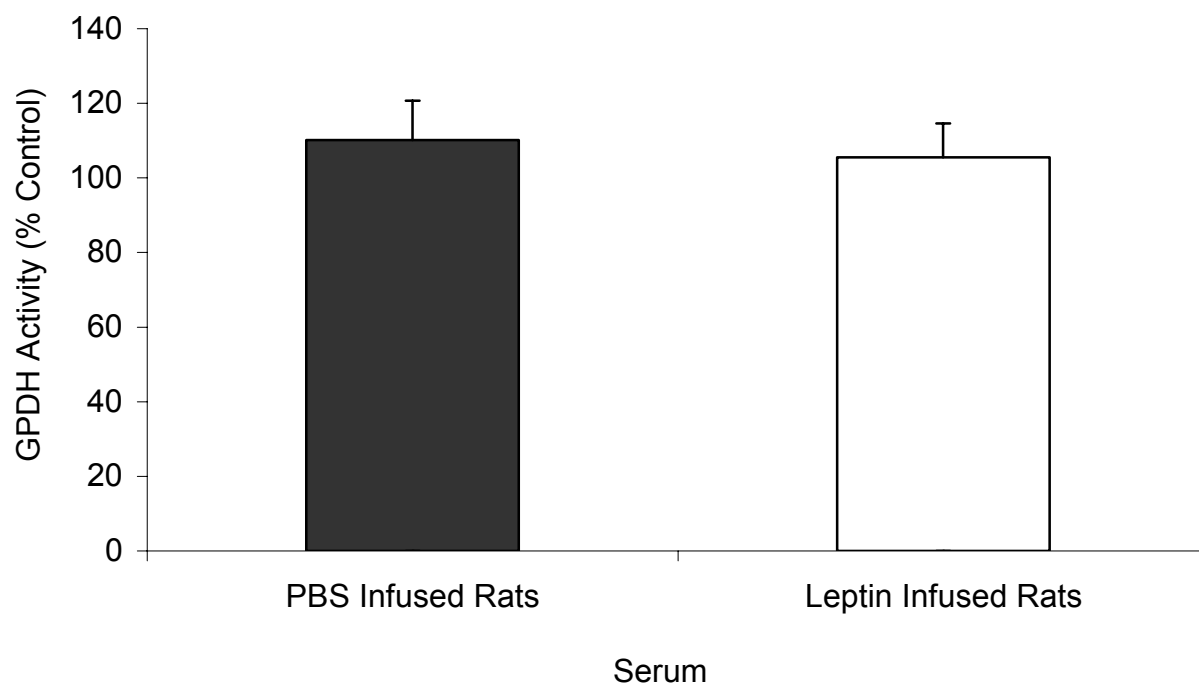
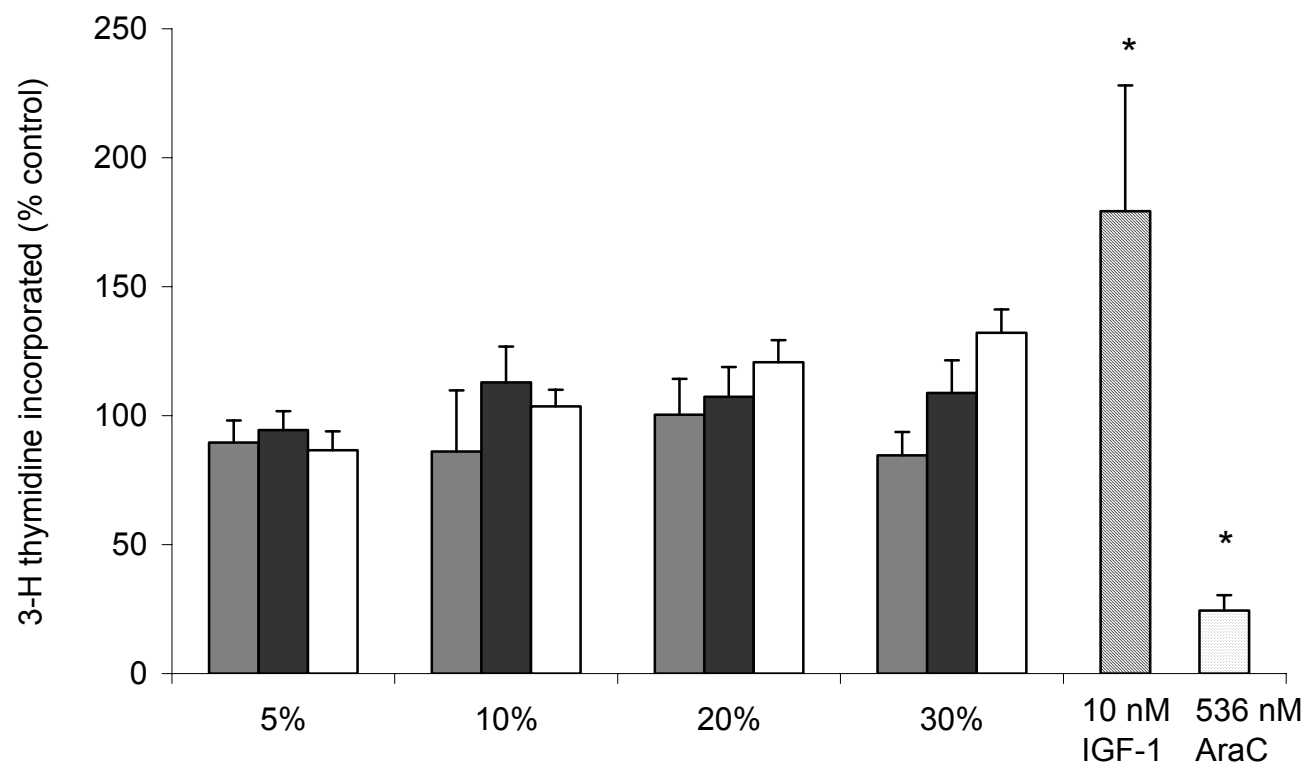


Figure 7



Adipose Tissue Conditioned Media (Concentration by Volume)

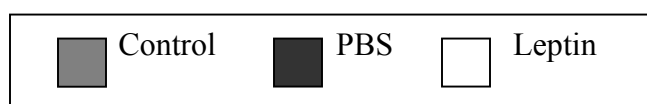


Figure 8

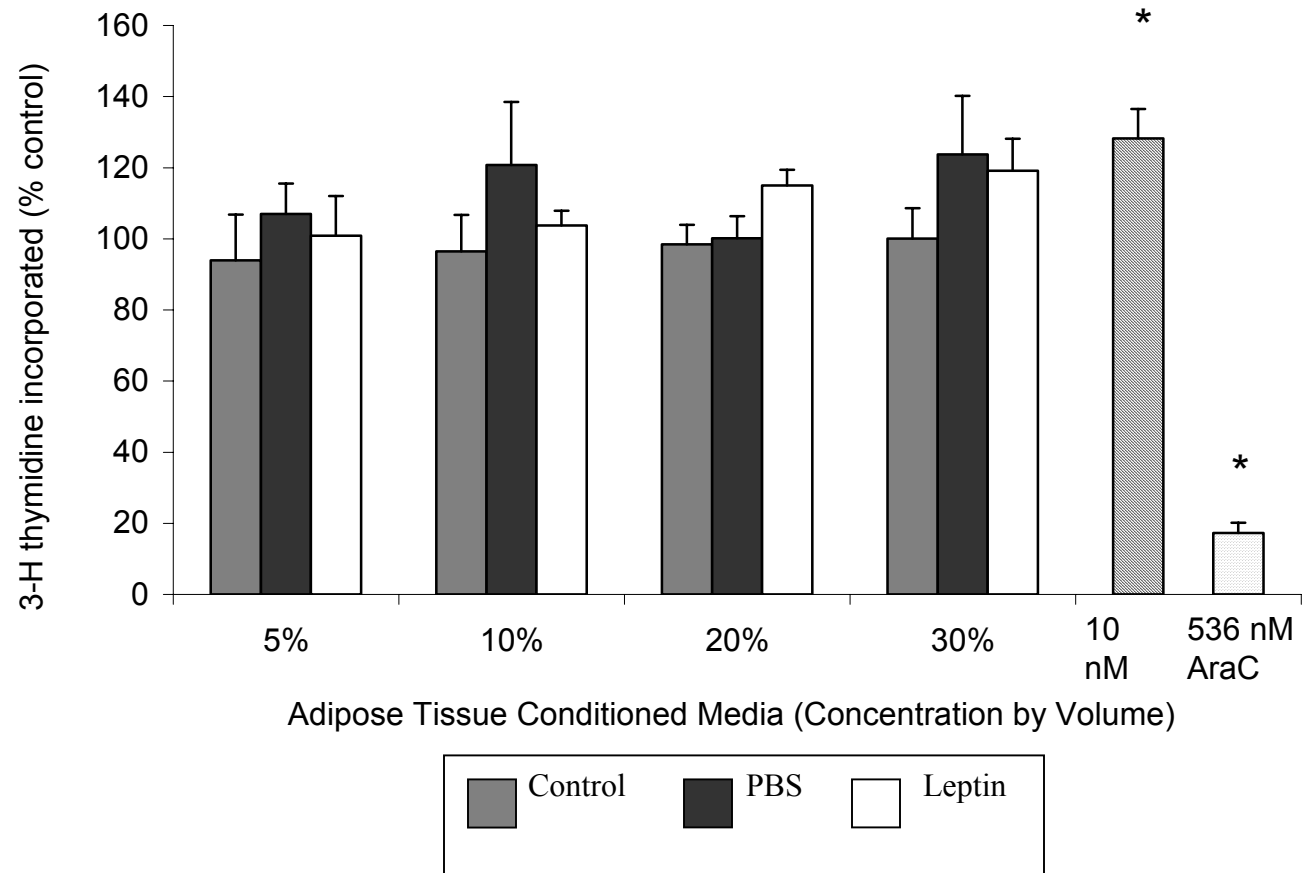
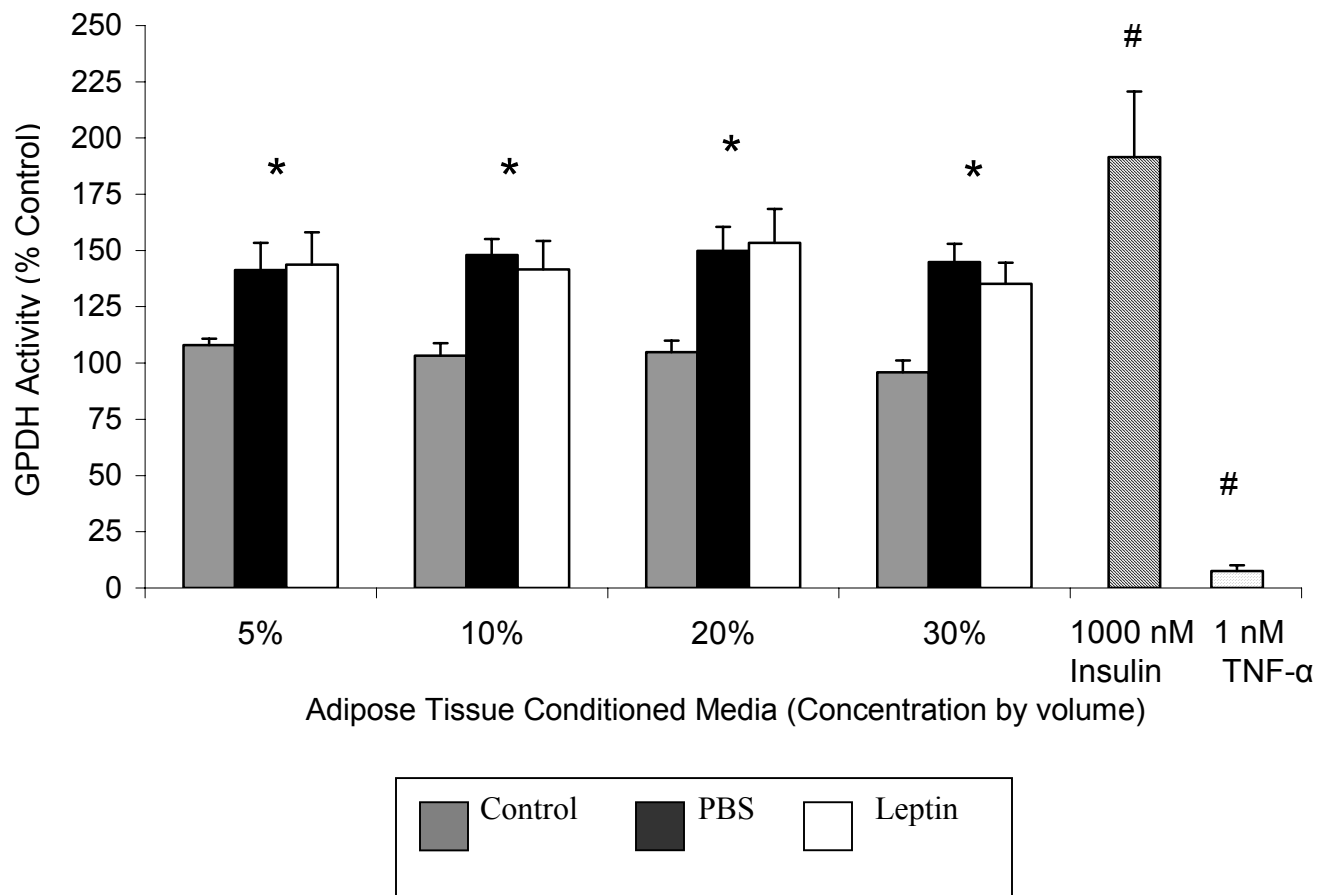


Figure 9

Preadipocyte Differentiation: Conditioned Media Dose Response



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CHAPTER 4

CONCLUSIONS

The studies described in this thesis illustrate the complexity of leptin's role in regulating body weight. The purpose of the studies was to determine whether leptin influences preadipocyte development by acting directly on preadipocytes, which express leptin receptors [1], or indirectly by modifying the release of another factor which then acts on the cells. We found that leptin modified preadipocyte proliferation through both direct and indirect mechanisms. High physiological levels of leptin (50 ng/ml) stimulated preadipocyte proliferation, which is not surprising since leptin has been shown to stimulate proliferation of various other cell types [2]. The physiologic relevance of this finding is unclear since the dose of leptin that stimulated preadipocyte proliferation is much higher than circulating concentrations of the protein *in vivo*, but could reflect local levels of leptin in adipose tissue, especially in obese animals since leptin expression is positively correlated with adipocyte size [3]. This observation also is consistent with results from *in vitro* studies that show that supraphysiologic doses of leptin are required to stimulate lipolysis and inhibit lipogenesis [4-5].

In contrast to leptin, low concentrations of serum from leptin infused rats inhibited proliferation of both preadipocyte and stromal vascular cell fractions, suggesting that leptin can inhibit development of adipose tissue indirectly by activating or inhibiting another factor that regulates preadipocyte proliferation. Since adipose tissue conditioned media from leptin infused rats did not affect preadipocyte or stromal vascular proliferation, it can be assumed that the factor is not released from fat. Additional studies

are needed to determine which factor leptin activates or inhibits. Potential factors include α -MSH and IL-15. Leptin stimulates the secretion of both factors [6-7], and IL-15 has been shown to decrease lipogenesis and increase lipolysis *in vitro* and *in vivo* [8-9], while peripheral infusion of α -MSH results in loss of body fat [6]. It is also possible that leptin stimulates or inhibits a novel factor that suppresses preadipocyte proliferation.

None of the treatments used in these studies modified preadipocyte differentiation. This is surprising since differentiation is the lipid filling of preadipocytes and leptin has been shown to decrease fatty acid synthesis [10-11] and lipid stores in mature adipocytes [12]. The difference in maturity of preadipocytes and adipocytes could explain why leptin did not affect preadipocyte differentiation. Preadipocytes may lack certain receptors needed to respond to differentiation stimuli. Adipose tissue conditioned media from both leptin and PBS infused rats increased preadipocyte differentiation, implying that adipose tissue releases a factor that stimulates lipid filling. This effect was not specific to leptin and we excluded the possibility of lipid filling being driven by availability of fatty acids or triglycerides, therefore, it is likely that one of the many proteins that are known to be secreted by adipocytes acts as a paracrine factor to increase the number of fat cells available for lipid storage.

Unlike rodents, obese humans are not leptin deficient, but instead have high levels of the protein, which implies that they are leptin resistant [13-14]. Nevertheless, the studies described here provide important insights into leptin's role in preadipocyte development. Discovery of the factor that leptin activates and/or inhibits to decrease preadipocyte proliferation will lead to a better understanding of fat cell development, and hopefully contribute to the search for effective treatment of obesity.

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