Direct and Indirect Effects of Leptin on Preadipocyte Proliferation and Differentiation

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ABSTRACT

Leptin has been shown to reduce body fat in vivo. Adipocytes express the leptin receptor, therefore it is realistic to expect a direct effect of leptin on adipocyte growth and metabolism. 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 tested the effect of leptin (0-500 ng/ml), serum from leptin infused rats (0.25% by volume), and adipose tissue 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 (P< 0.05), but 250 and 500 ng leptin/ml inhibited proliferation of both preadipocyte and stromal vascular cell fractions (P< 0.01), as measured by 3H-thymidine incorporation. Serum from leptin infused rats inhibited proliferation of the adipose and stromal vascular (P = 0.01) fractions, but adipose tissue 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.

KEYWORDS: proliferation, differentiation, adipogenesis, cell culture
INTRODUCTION

Parabiosis studies in genetically obese mice indicate that there is a circulating factor involved in the regulation of food intake and body weight (12, 24). Leptin, a circulating hormone produced by the obese (ob) gene in adipose tissue (63), has been shown experimentally to reduce body weight by decreasing food intake and increasing energy expenditure in rodents (45, 22). The reduction in body weight is specific to fat, as there is no loss of lean mass in leptin-treated animals (45).

Leptin initiates biological responses by binding to its receptor, Ob-R, which is found in the brain and most peripheral tissues, including white adipose tissue (55). Direct injections of leptin into the brain of rodents decrease appetite and body fat (11), so it was initially thought that leptin exerted its effects exclusively by binding receptors in the central nervous system (55). Peripheral infusions of leptin in mice also reduce body weight and body fat content (27) and this can be achieved without producing a significant reduction in food intake (9). Some of the responses to peripherally administered leptin may be mediated by leptin crossing the blood brain barrier and activating leptin receptors in brain that are associated with the control of food intake or energy metabolism, but others may be dependent upon activation of leptin receptors in peripheral tissue (see (7) for review).

The specific pathways that are responsible for the loss of fat in leptin-treated animals have not been fully elucidated and more attention has been paid to changes in lipid metabolism of mature fat cells than responses made by preadipocytes that are destined to become fat cells. Studies examining metabolism in mature, lipid-filled fat cells have shown that leptin stimulates adipose tissue lipolysis both in vitro (21, 60) and in vivo (20), stimulates fatty acid oxidation (64) and inhibits insulin-stimulated lipogenesis both in vivo (23) and in vitro (5) but does not
inhibit basal lipogenesis \textit{in vivo} (23). The \textit{in vitro} studies indicate that leptin has the potential to directly activate adipose tissue leptin receptors, however, very high concentrations of leptin are required to stimulate lipolysis \textit{in vitro} (21) compared with those that are effective \textit{in vivo} (20) which suggests the involvement of additional factors \textit{in vivo} that either enhance or mediate the leptin response. \textit{In vivo}, leptin may stimulate lipolysis in adipocytes directly or indirectly either by inducing release of another circulating factor or by activating the sympathetic nervous system. Several studies have shown that leptin can activate sympathetic outflow to tissues such as brown adipose tissue (51) and the kidneys (30) but it is not yet clear whether there is a biologically significant stimulation of sympathetic activity in white adipose tissue (13).

In addition to increasing lipolysis and fatty acid oxidation and decreasing insulin-stimulated lipogenesis to reduce the size of mature, lipid-filled fat cells leptin may affect the size of white adipose depots by inhibiting proliferation and/or differentiation of preadipocytes. Only two studies have examined the effect of leptin on preadipocyte proliferation (36, 47). One study showed that 160 ng/ml murine leptin increased proliferation in a primary culture of rat stromal vascular cells, but stromal vascular cells were not separated from adipocytes, so it is uncertain whether proliferation could be attributed to preadipocytes, stromal vascular cells or both (36). Ramsay (47) found increased proliferation in porcine preadipocytes treated with 1,000 ng/ml porcine leptin. The large difference in the dose required to increase proliferation in the two studies could be due to the different sources of cells and leptin or due to the cell type responsible for the proliferation. Three studies have examined the effect of leptin on preadipocyte differentiation \textit{in vitro} (36, 47, 56). One study reported an inhibition of differentiation of preadipocytes derived from a human marrow stromal cell line (56), the second study reported an increase in differentiation of preadipocytes derived from white fat obtained from male Sprague-
Dawley rats (36) and the third study found no effect of leptin on differentiation of porcine preadipocytes (47).

The purpose of the studies described here was to determine whether leptin affected proliferation and/or differentiation of preadipocytes derived from rat adipose tissue and, if there was an effect, whether it is due to a direct action of leptin on the cells or whether it was mediated by another circulating factor originating in adipose tissue.

MATERIALS AND METHODS

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Georgia and were conducted according to American Physiology Society Guiding Principles in the Care and Use of Animals (3).

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 in detail previously (48). Briefly, rats were anesthetized with ketamine (200mg/kg body weight i.p.) and xylazine (10mg/kg i.p.) before removal of inguinal fat pads in sterile conditions. Fat was minced with scissors and incubated with shaking in digestion buffer (HEPES containing 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 Chemical Co., St. Louis, MO) containing antibiotics (50 mg/L gentamycin sulfate, 50 mg/L cefazolin, 2.5 mg/L amphotericin) and centrifuged at 350 x g 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 plating medium (DMEM/F12, antibiotics, 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. Cells were seeded at a density of $4.8 \times 10^3$ cells/cm$^2$ on 12.5 cm$^2$ tissue culture flasks for the proliferation assay and at a density of $1 \times 10^4$ cells/cm$^2$ on 35 mm dishes for the differentiation assay. Cells were maintained at 37°C and 5% humidity for the duration of the assays.

**Proliferation Assay**

Twenty four hours after plating (*Day 1*), plating media was replaced with serum free media for 24 hours. On *Day 2*, 2 ml of test media consisting of DMEM/F12 media, antibiotics, and 0.25% control rat serum (pooled serum collected from ad libitum fed rats) by volume was added to cells. This media served as a control media and a basal media to which treatments were added, as described below for Experiments 1 and 2. On *Day 4* treatment media was replaced with fresh treatment media containing $^3$H-thymidine (0.5µCi/flask). Twenty-four hours later, $^3$H-thymidine was removed with cold thymidine rinse media (12 mg thymidine/1000ml DMEM). Cells were placed on lipid filling media (DMEM/F12, antibiotics, 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 SV cells by density gradient centrifugation using a Percoll gradient, as described by Novakofski (43). Each cell fraction was transferred to a scintillation vial and incorporation of $^3$H-thymidine into SV cells or adipocytes was measured by scintillation counting. Data was calculated as pmol of thymidine incorporated per flask and expressed as percent of the control.
**Differentiation Assay**

Twenty-four hours after plating (Day 1), plating media was removed and cells were incubated in serum free media for 24 hours. On Day 2, test media containing serum free DMEM/F12, antibiotics, 10 nM insulin, 60 nM tranferrin, and 2 nM thyroxine was added to cells. This served as a control and a basal media to which treatments were added, as described below for Experiments 1 and 2. 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 (61) and modified by Ramsay et al (48). Protein concentration was measured using a kit (Bio-Rad, Melville, New York). GPDH results were calculated as mUnits/min/mg protein and results were expressed as a percent of the control.

**Conditioned Media and Serum from Leptin Treated Rats**

Twenty two 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 from 7:00 a.m., and free access to chow (Purina Rodent chow 5001: Purina Mills, St. Louis, MO). After a 7 day acclimation period, daily food intakes and body weights were recorded. After 3 days of baseline measures, rats were weight matched into two groups and fitted with intraperitoneal Alzet miniosmotic pumps (Direct Corp. Cupertino, CA Model 1007D) which delivered either 100 µg recombinant rat leptin (R&D Systems, Minneapolis, MN) in PBS or an equivalent volume of PBS over 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 concentrations were 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 (38). Cell number was subsequently used to adjust the volume of the conditioned media to normalize volume based on the number of cells that were present in the incubated fat. Large blood vessels were removed from the remaining fat, which was minced and washed 3-4 times with 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 also was incubated and used as a control in the cell culture assays, as described below. Following the incubation period, conditioned media and DMEM/F12 were filtered through Fisherbrand P8 filter paper. The filtered media was then sterile filtered (Steriflip Filters, 0.22 µm), frozen at -80°C and later diluted with varying amounts of DMEM/F12 to equalize volume based on cell number counts.

Experiment 1

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 both the proliferation and differentiation assays consisted of varying concentrations of leptin (5, 50, 100, 250, 500 ng/ml) added to the basal control medium described above. AraC (536 nM), a DNA synthesis inhibitor, was used as a negative control for preadipocyte proliferation and 10 nM
IGF-1 was used as a positive control. TNF-α (1 nM) was used as a negative control for preadipocyte differentiation and 1000 nM insulin was used as a positive control.

**Lactate Dehydrogenase Assay**

On Days 4 and 5 of the proliferation assay (days 2 and 3 of exposure to treatments), media from cells treated with 100 ng/ml leptin or 536 nM AraC was collected and assayed for lactate dehydrogenase (LDH) using a kit (Sigma DG1340-K: Sigma Chemical Co.) according to the manufacturers protocol. This provided a measure of cell damage.

**Experiment 2**

Basal test medium for both the proliferation and differentiation assays was supplemented with 0.25% serum by volume from each of the leptin- and PBS-infused rats. Treatment serum replaced the control rat serum in the proliferation assay. In addition, various concentrations by volume of conditioned media (5, 10, 20, or 30%) were added to basal test medium. Each conditioned media treatment was diluted to be equivalent of having been exposed to 150,000 adipocytes/ml before it was added to the basal medium. 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. DMEM/F12 media that had not been exposed to adipose tissue (5, 10, 20, or 30% by volume) was used as a control. Conditioned media was analyzed for free fatty acid (NEFA C Kit, Wako Chemicals, GMbH, Germany) and triglyceride (L-Type TG-H Kit, Wako Chemicals) concentrations according to the manufacturer’s protocols. The leptin concentrations of the media also was measured and found to be at, or below, the lowest detectable level of the assay (0.5 ng/ml: data not shown).
**Statistical Analysis**

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 SV 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 SV 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 proliferation \( (P < 0.01) \): Figure 1A). Similarly, 250 and 500 ng/ml leptin significantly decreased SV cell proliferation \( (P < 0.01) \): Figure 1B). As expected, IGF-1 stimulated proliferation of both the adipose and SV fractions, by 49 and 23% respectively, compared to control (Figures 1A and 1B). AraC decreased preadipocyte and SV cell proliferation by 45 and 83% respectively compared with control (Figures 1A and 1B). Leptin had no effect on preadipocyte differentiation as measured by GPDH activity (data not shown), whereas 1 nM TNF-\( \alpha \) decreased GPDH activity by 59% and 1000 nM insulin increased it by 59% compared with control.
Media collected from cultures exposed to 100 ng/ml leptin or AraC treatments had significantly less LDH activity than the lipid calibrator provided by the manufacturer (Figure 2), indicating that the inhibition of proliferation in cells treated with leptin or AraC was not associated with substantial levels of cell death.

**Experiment 2**

There was no significant difference in beginning body weight, end body weight, food intake or epididymal, retroperitoneal or perirenal fat pad weights between rats infused with 100 ug leptin per day or with PBS for 5 days (Table 1). Leptin infused rats gained significantly less weight during infusion, had smaller mesenteric fat pads (P < 0.05) and had significantly higher serum leptin levels on both days 3 and 5 of infusion compared with PBS infused rats (P < 0.01: Table 1). The control rat serum, which was used in the test media for the proliferation assay, 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 = 0.01) and SV cells (P = 0.01) compared with serum from PBS-infused rats (Figure 3) but did not effect preadipocyte differentiation (data not shown). There was no difference in preadipocyte or SV cell proliferation or in preadipocyte differentiation of cells exposed to adipose tissue conditioned media from leptin- or PBS-infused rats, and there was no interaction between volume and source of media on proliferation (adipose tissue conditioned media or control conditioned media) (Table 2). In contrast, all concentrations of adipose conditioned media significantly increased preadipocyte differentiation compared with conditioned media not exposed to adipose tissue (Table 3). There were no differences in the FFA or triglyceride concentrations of the conditioned media from PBS- and leptin-infused rats (FFA: 0.10 ± 0.01 and 0.09 ± 0.01ng/ml for PBS and
leptin-infused conditioned media respectively. Triglycerides: 32 ± 5 and 31 ± 7 mEq/L for PBS and leptin-infused conditioned media respectively). IGF-1 and AraC significantly increased and decreased preadipocyte and SV proliferation, respectively (P < 0.05: data not shown). Preadipocyte differentiation was significantly increased by 1000 nM insulin and decreased by 1 nM TNF-α (data not shown).

DISCUSSION

In this experiment, we examined whether leptin modified preadipocyte development (proliferation and/or differentiation) directly and/or indirectly. Leptin alone had a biphasic effect on preadipocyte proliferation. A relatively low concentration of 50 ng/ml leptin significantly increased preadipocyte proliferation, whereas 250 and 500 ng/ml leptin significantly inhibited both preadipocyte and stromal vascular proliferation. In contrast, very low concentrations of serum from leptin-infused rats decreased proliferation of both adipose and stromal vascular cell fractions compared with serum from PBS-infused rats. These results imply that leptin can modify the rate of growth of adipose depots indirectly, possibly by activating or inhibiting another circulating factor, to decrease proliferation. Although 0.25% by volume serum from leptin-infused rats decreased preadipocyte and SV proliferation, concentrations of up to 30% by volume of adipose tissue conditioned media had no effect on cell proliferation, indicating that the active inhibitory factor is unlikely to be released from white fat. In contrast, although 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 at least one factor released from fat that stimulates preadipocyte differentiation. It also is possible that the increased differentiation was due to
increased availability of fatty acids and triglycerides, but this is unlikely because the response was not dose dependent.

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 (37). In addition, other investigators have reported that leptin increases preadipocyte proliferation in primary culture (36, 47). Adipocytes express the leptin receptor (53) making it possible for leptin to act directly on adipocytes and preadipocytes to regulate growth and metabolism. The amount of leptin required to stimulate proliferation in preadipocytes \textit{in vitro} was higher that 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 (44) exceeding concentrations of 50 ng/ml in genetically obese animals (25). Similar circulating concentrations of leptin have been reported for humans (14). The concentration of leptin in extracellular fluid surrounding adipocytes may be significantly higher than that in the circulation, thus leptin could function as a paracrine regulator of preadipocyte proliferation in fat depots. Expression of leptin mRNA varies across different fat depots and correlates with fat cell size (37, 26). In rats leptin mRNA expression is higher in retroperitoneal and parametrial fat than in perirenal or inguinal fat (26), but does not correlate with the propensity of different fat depots to enlarge by proliferation versus lipid filling of existing adipocytes (16). All depots, however, tend to show increased rates of proliferation after existing adipocytes fill with lipid (28) and one could speculate that increased leptin secretion from the enlarged adipocytes contributes to the promotion of preadipocyte proliferation as obesity develops. The media used in the proliferation assay provided an abundance of nutrients, and contained high concentrations of insulin, which are similar to baseline concentrations in conditions of obesity or to post-ingestive concentrations in
normal weight animals. The 50 ng/ml leptin concentration may have provided optimal conditions for proliferation when coupled with the insulin and nutrients available in the media. Thus, in conditions of positive energy balance in non-obese animals the associated increase in leptin concentration may contribute to enlargement of white fat depots by increasing the number of cells available for lipid accumulation. Circulating concentrations of leptin are approximately 9-fold higher in obese than normal individuals (14), which means that leptin in the extracellular fluid bathing adipocytes would be even higher. Thus this might represent a condition in which very high concentrations of leptin inhibit preadipocyte proliferation, but, because white fat cells are leptin resistant in conditions of extreme obesity (59), the potential for leptin to inhibit preadipocyte or adipocyte growth or metabolism may be irrelevant.

We did not investigate the mechanistic basis of inhibition of proliferation by very high concentrations of leptin (250 and 500 ng/ml). It is possible, however, that the transition from stimulation to inhibition of proliferation as leptin concentration increased represented recruitment of different subtypes of the leptin receptor. A different explanation is that the high concentrations of leptin nonspecifically bound to other cytokine receptors to inhibit proliferation, especially since the inhibition was not limited to preadipocytes. Alternatively, high levels of leptin could interfere with insulin signaling. 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 (41). Insulin is a mitogenic factor and has been shown to increase proliferation and differentiation of preadipocytes in cell lines (52, 54). 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) (7), therefore, high
concentrations of leptin could activate these pathways and interfere with 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 have been shown to increase apoptosis of adipocytes (46), so it cannot be ruled out that higher doses of leptin caused cell death in this study, however, 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 (47). Serum and adipose tissue conditioned media from leptin-infused rats did not affect preadipocyte differentiation compared to serum or conditioned media from PBS-infused rats. As expected, positive and negative controls increased and decreased preadipocyte differentiation respectively, therefore if leptin alone, serum from leptin-infused animals, or adipose tissue conditioned media from leptin-infused animals induced a change in preadipocyte differentiation, the response would have been detected. This negative result was somewhat surprising since leptin has been shown to reduce the size of mature adipocytes by inhibiting insulin-stimulated lipogenesis (23, 5) and by increasing lipolysis (20, 21) and fatty acid oxidation (64). Recent studies have clarified the post-receptor signaling events and involvement of specific transcription factors involved in the mediation of different aspects of leptin activity (6, 19, 42), including changes in lipid and glucose metabolism of mature adipocytes (34, 39), but little is known about the effect of leptin on preadipocyte proliferation. It is likely that early stages of the signaling processes that mediate changes in rates of preadipocyte proliferation are similar to those in other tissue types and are associated with activation of MAPK (35, 8). In this study we used a primary culture of SV cells, therefore, differences in metabolism of preadipocytes compared with differentiated adipocytes could explain why we did not see an effect of leptin on the lipid filling of preadipocytes. The SV cells used in this study were obtained from inguinal fat which is a
depot that tends to enlarge during aging by hyperplasia rather than hypertrophy (16) and it is possible that SV cells derived from other fat depots, such as epididymal or mesenteric fat, which enlarge by hypertrophy (16), would have shown less of a proliferative response to leptin. Consistent with this notion, in previous studies we have found that peripheral infusions of leptin cause reliable changes in the size of both inguinal and retroperitoneal fat depots in rats but have a smaller impact on the size of epididymal and mesenteric fat (49). Further experiments are needed to determine whether the effect of leptin on preadipocyte proliferation is depot specific because the two other studies that have reported an effect of leptin on preadipocyte proliferation also used cells from subcutaneous adipose tissue (36, 47).

In the second study reported here low concentrations of serum from leptin-infused rats significantly inhibited proliferation of both preadipocytes and SV cells. The results from the first experiment and the fact that the control pool of serum used for the basal media contained more leptin than the test media from leptin-treated rats confirmed that high serum leptin concentrations could not account for the inhibition of proliferation. These data imply that leptin treatment results in the release, or inhibition, of another metabolically active circulating factor which influences preadipocyte growth. These results are consistent with observations that leptin increases endothelial cell proliferation in vitro (8), but decreases proliferation in vivo (50), implying that there are additional factors involved in the in vivo response to leptin. Although leptin has been reported to stimulate sympathetic outflow to some tissues (29) and that increased sympathetic activity inhibits adipocyte proliferation (10), this mechanism cannot account for changes in cell number in an in vitro condition. It is possible, however, that leptin stimulated the release of norepinephrine, which has been shown to inhibit preadipocyte proliferation (32). This is not likely the factor responsible for decreased proliferation in this study because
norepinephrine becomes unstable once blood is collected unless preservatives are added (15). Many studies have examined the relationship between leptin and other cytokines, hormones, and factors secreted from non-adipose tissues (58). TNFα is produced by non-adipocyte cells in adipose tissue and is increased in tissue from obese individuals (17). Although TNFα has been shown to inhibit preadipocyte proliferation in primary rat preadipocyte culture (33) it is released extremely slowly from adipose tissue into the circulation (40) and there is no correlation between circulating concentrations of leptin and TNFα in humans (4), making it unlikely that this cytokine was responsible for the inhibitory effect of serum from leptin-infused rats on preadipocyte proliferation. 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 (1). IL-15 and it’s 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 (1, 2). These metabolic responses to IL-15 administration are very similar to those caused by leptin administration. Leptin has been shown to increase IL-15 activation of NK cells in vitro (57). Therefore, it is possible that leptin activates a factor such as IL-15 to inhibit preadipocyte proliferation. Another factor that may be involved is alpha melanocyte stimulating hormone (αMSH). Recent studies have shown significant weight loss in ob/ob mice treated with an αMSH analog (18) and that leptin administration doubles circulating concentrations of αMSH in these mice (31). In vitro, αMSH decreases adipocyte leptin expression and secretion (31). Therefore, a possible feedback loop exists between αMSH and leptin, in which leptin increases αMSH secretion and αMSH downregulates leptin expression and secretion.
The source of the serum factor responsible for decreasing proliferation is unknown. Leptin is produced by adipose tissue in proportion to the amount of fat present (62) and reduces body fat mass in experimental animals (45), so it is reasonable to assume that the factor might be produced by adipocytes. In Experiment 2, however, we found no difference in preadipocyte or SV proliferation in cells treated with conditioned media from leptin- or PBS-infused rats or with non-adipose control conditioned media, suggesting that leptin does not activate a factor released from fat to decrease proliferation. Alternatively, the serum factor may have been derived from adipose tissue but was present at low concentrations in conditioned media. This should have been, at least partially, accounted for by the 120-fold higher concentrations of conditioned media tested (30% by volume) compared with the effective concentrations of serum (0.25% by volume). It also is possible that the four hour exposure of media to adipose tissue did not allow enough time for the factor to be secreted from adipose tissue. In contrast to the absence of any effect on proliferation, adipose tissue conditioned media from both leptin- and PBS-infused rats significantly increased preadipocyte differentiation, implying that a factor released from fat promotes lipid filling. Fatty acids and triglycerides were present at low levels in conditioned media, therefore they could not account for the increase in lipid filling of preadipocytes. Other factors released from fat, such as IGF-1, may have been responsible for increased preadipocyte differentiation (28), but we did not measure the IGF-1 concentrations in the conditioned media.

In summary, the studies described here demonstrate that leptin can act directly on adipose tissue to increase preadipocyte proliferation and indirectly to inhibit preadipocyte and SV proliferation. The inhibition appears to be due to the activation or inhibition of an additional circulating factor and further studies are needed to identify this factor. Leptin had no direct or indirect effect on preadipocyte differentiation, despite evidence for inhibition of insulin-
stimulated lipogenesis and increased lipolysis on mature adipocytes. The results of these studies reinforce the complexity of leptin action, but provide new insights into its role in preadipocyte development.
ACKNOWLEDGEMENTS

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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.

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>Leptin</th>
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<tbody>
<tr>
<td>Start body weight (g)</td>
<td>340 ± 6</td>
<td>339 ± 5</td>
</tr>
<tr>
<td>End body weight (g)</td>
<td>344 ± 6</td>
<td>336 ± 5</td>
</tr>
<tr>
<td>Weight change (g/5 day)</td>
<td>3.6 ± 2.0</td>
<td>-2.8 ± 2.4 *</td>
</tr>
<tr>
<td>Food intake (g/ day)</td>
<td>19.5 ± 2</td>
<td>18.0 ± 1</td>
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<tr>
<td>Epididymal Fat (g)</td>
<td>2.7 ± 0.1</td>
<td>2.9 ± 0.3</td>
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<tr>
<td>Retroperitoneal Fat (g)</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Perirenal Fat (g)</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.1</td>
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<tr>
<td>Mesenteric Fat (g)</td>
<td>1.5 ± 0.1</td>
<td>1.0 ± 0.2 *</td>
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<tr>
<td>Serum leptin Day 3 (ng/ml)</td>
<td>1.7 ± 0.2</td>
<td>5.3 ± 1.3 *</td>
</tr>
<tr>
<td>Serum leptin Day 5 (ng/ml)</td>
<td>1.2 ± 0.2</td>
<td>3.2 ± 0.6 *</td>
</tr>
</tbody>
</table>

Values are means ± sem for 6-9 rats in the leptin group and 7-10 in the PBS group. An asterisk indicates a significant difference (P < 0.05) between leptin and PBS infused rats.
Table 2. Incorporation of $^3$H thymidine (% of control) into the preadipocyte and SV cell fractions in response to adipose tissue conditioned media and control conditioned media.

<table>
<thead>
<tr>
<th>Conditioned Media Source</th>
<th>Volume</th>
<th>Control</th>
<th>PBS</th>
<th>Leptin</th>
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<tbody>
<tr>
<td><strong>Preadipocyte Fraction</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>108 ± 13</td>
<td>107 ± 9</td>
<td>101 ± 11</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>94 ± 10</td>
<td>121 ± 18</td>
<td>104 ± 4</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>96 ± 5</td>
<td>100 ± 6</td>
<td>115 ± 4</td>
</tr>
<tr>
<td></td>
<td>30%</td>
<td>98 ± 9</td>
<td>124 ± 16</td>
<td>119 ± 9</td>
</tr>
<tr>
<td><strong>SV Fraction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>89 ± 8</td>
<td>94 ± 97</td>
<td>87 ± 7</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>86 ± 24</td>
<td>113 ± 14</td>
<td>104 ± 6</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>100 ± 14</td>
<td>107 ± 11</td>
<td>121 ± 9</td>
</tr>
<tr>
<td></td>
<td>30%</td>
<td>85 ± 9</td>
<td>109 ± 13</td>
<td>132 ± 9</td>
</tr>
</tbody>
</table>

Data are means ± sem (n= 6-7 rats), with each sample tested 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 (adipose tissue conditioned media or control conditioned media).
Table 3. GPDH activity (% control) as affected by varying concentrations of adipose tissue conditioned media and control conditioned media.

<table>
<thead>
<tr>
<th>Conditioned Media Source</th>
<th>Volume</th>
<th>Control</th>
<th>PBS</th>
<th>Leptin</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>5%</td>
<td>108 ± 3 *</td>
<td>141 ± 12</td>
<td>144 ± 14</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>103 ± 6 *</td>
<td>148 ± 7</td>
<td>142 ± 13</td>
</tr>
<tr>
<td></td>
<td>20%</td>
<td>105 ± 5 *</td>
<td>150 ± 11</td>
<td>153 ± 15</td>
</tr>
<tr>
<td></td>
<td>30%</td>
<td>96 ± 5 *</td>
<td>145 ± 8</td>
<td>135 ± 9</td>
</tr>
</tbody>
</table>

Data are means ± sem (n= 6-7 rats), with each sample tested in triplicate. There was no difference in differentiation within or between leptin- and PBS-infused groups. An asterisk indicates that there was a significant difference (P < 0.05) between adipose tissue conditioned media and control media not exposed to adipose tissue.
REFERENCES


Proliferation of preadipocyte (A) and stromal vascular (B) cells treated with varying concentrations of leptin. Data represents mean ± sem for 3 to 6 replicate cultures. Superscripts denote significant effects of leptin on proliferation (P< 0.05). An asterisk represents a significant difference between positive or negative controls compared to the 0 ng/ml leptin condition.
Cell viability as measured by LDH activity in cell culture media. Data are means ± sem for 4 to 6 replicates, each determined in quadruplicate. An asterisk represents significantly (P<0.05) lower LDH activity in media collected from cells treated with 100 ng/ml leptin or AraC for 2 or 3 days compared with a lipid calibrator control provided by the manufacturer.
Incorporation of $^3$H-thymidine into the preadipocyte and SV cell fractions treated with 0.25% serum from rats infused with PBS or leptin. Data represents mean ± sem for 6-10 rats, with each treatment determined in triplicate. An asterisk indicates a significant difference between cells exposed to serum from PBS-infused and leptin-infused rats (P<0.01)