Direct and indirect effects of leptin on preadipocyte proliferation and differentiation

Blair Wagoner, Dorothy B. Hausman, and Ruth B. S. Harris

Department of Foods and Nutrition, University of Georgia, Athens, Georgia

Wagoner, Blair, Dorothy B. Hausman, and Ruth B. S. Harris. Direct and indirect effects of leptin on preadipocyte proliferation and differentiation. *Am J Physiol Regul Integr Comp Physiol* 290: R1557–R1564, 2006. First published January 19, 2006; doi:10.1152/ajpregu.00860.2005.—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 supraphysiological doses of the protein to see a decrease in lipogenesis or stimulation of lipolysis, implying an indirect action of leptin. It also is 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 medium 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 fractions ($P = 0.01$), but adipose tissue-conditioned medium had no effect on proliferation of either cell fraction. None of the treatments changed preadipocyte differentiation as measured by *sn*-glycerophosphate dehydrogenase activity. These results suggest that leptin could inhibit preadipocyte proliferation by modifying release of a factor from tissue other than adipose tissue.

adipogenesis; cell culture

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, because 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 on activation of leptin receptors in peripheral tissue (see Ref. 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 to 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) and stimulates fatty acid oxidation (64) and inhibits insulin-stimulated lipogenesis both in vivo (23) and in vitro (5) but does not inhibit basal lipogenesis in vivo (23). The 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 in vitro (21) compared with those that are effective in vivo (20), which suggests the involvement of additional factors in vivo that either enhance or mediate the leptin response. In vivo, leptin may stimulate lipolysis in adipocytes directly or indirectly by either inducing release of another circulating factor or 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 to the cell

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type responsible for the proliferation. Three studies have examined the effect of leptin on preadipocyte differentiation 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 present studies 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 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 the American Physiology Society's "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 (200 mg/kg body wt ip) and xylazine (10 mg/kg ip) before removal of inguinal fat pads in sterile conditions. Fat was minced with scissors and incubated with shaking in digestion buffer (HEPES containing 1,000 U/ml collagenase) for 90–120 min 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)/F-12 Ham's medium (Sigma, St. Louis, MO) containing antibiotics (50 mg/l gentamycin sulfate, 50 mg/l cefazolin, and 2.5 mg/l amphotericin) and centrifuged at 350 *g* for 10 min 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, Minneapolis, MN) was added to SV cells for 5 min, after which SV cells were resuspended in plating medium (DMEM/F-12, antibiotics, 10% FBS) and centrifuged at 1,800 rpm for 5 min. A small aliquot of SV cells was diluted with Rappaport's stain and counted on a hemocytometer. Cells were seeded at a density of 4.8×10^3 cells/cm² on 12.5-cm² tissue culture flasks for the proliferation assay and at a density of 1×10^4 cells/cm² 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 medium was replaced with serum-free medium for 24 h. On *day 2*, 2 ml of test medium consisting of DMEM/F-12 medium, antibiotics, and 0.25% control rat serum (pooled serum collected from ad libitum-fed rats) by volume was added to cells. This medium served as a control medium and a basal medium to which treatments were added, as described for *experiments 1* and *2*. On *day 4*, treatment medium was replaced with fresh treatment medium containing [³H]thymidine (0.5 μCi/flask). Twenty-four hours later, [³H]thymidine was removed with cold thymidine rinse medium (12 mg thymidine/1,000 ml DMEM). Cells were placed on lipid filling medium (DMEM/F-12, antibiotics, 10% pig serum, 1.0 nM insulin, and 10 U/ml heparin), which was changed every other day until *day 13*. On *day 14*, cells were enzymatically harvested using HBSS (Sigma H-1387; Sigma) containing 0.5% bovine serum albumin, 0.5 mg/ml trypsin, and 125 U/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 [³H]thymidine into SV cells or adipocytes was measured by scintillation counting.

Data were calculated as picomoles of thymidine incorporated per flask and expressed as a percentage of the control.

Differentiation assay. Twenty-four hours after plating (*day 1*), plating medium was removed and cells were incubated in serum-free medium for 24 h. On *day 2*, test medium containing serum-free DMEM/F-12, antibiotics, 10 nM insulin, 60 nM transferrin, and 2 nM thyroxine was added to cells. This served as a control and a basal medium to which treatments were added, as described for *experiments 1* and *2*. Fresh treatment medium 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, NY). GPDH results were calculated as milliunits per minute per milligram of protein, and results are expressed as a percentage of the control.

Conditioned medium and serum from leptin-treated rats. Twenty-two male Sprague-Dawley rats (Harlan Industries) weighing between 320 and 340 g were housed in hanging wire mesh cages at 23°C, with lights on 12 h/day from 7:00 AM 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 (model 1007D; Direct, Cupertino, CA), which delivered either 100 μg of recombinant rat leptin (R&D Systems, Minneapolis, MN) in PBS or an equivalent volume of PBS over 24 h for 5 days. Blood was collected on the third day of infusion by tail bleed for measurement of serum leptin (rat leptin RIA kit; Linco, St. Louis, MO). After 5 days of infusion, rats were killed, trunk blood was collected, and epididymal, retroperitoneal, perirenal, and mesenteric fat pads were dissected and weighed. Serum leptin concentrations were measured, and serum was added to the cell culture assays where indicated.

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 via a Coulter counter and multisizer as described previously (38). Cell number was subsequently used to adjust the volume of the conditioned medium 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, washed three to four times with 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/F-12 in 12.5-cm³ incubation flasks for 4 h at 37°C and 5% humidity. DMEM/F-12 containing no adipose tissue also was incubated and used as a control in the cell culture assays, as described below. After the incubation period, conditioned medium and DMEM/F-12 were filtered through Fisherbrand P8 filter paper. The filtered medium was then sterile filtered (Steriflip filters, 0.22 μm), frozen at −80°C, and later diluted with varying amounts of DMEM/F-12 to equalize volume based on cell number counts.

Experiment 1. Proliferation and differentiation assays were performed on six different pools (1 pool = 2 rats) of cells, and treatments were run in quadruplicate on each pool of cells. Treatment medium for both the proliferation and differentiation assays consisted of varying concentrations of leptin (5, 50, 100, 250, or 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 1,000 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), medium 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) according to the manufacturer's 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 medium (5, 10, 20, or 30%) were added to basal test medium. Each conditioned medium treatment was diluted to the equivalent of having been exposed to 150,000 adipocytes/ml before it was added to the basal medium. Serum and conditioned medium 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/F-12 medium that had not been exposed to adipose tissue (5, 10, 20, or 30% by volume) was used as a control. Conditioned medium was analyzed for free fatty acid (FFA; NEFA C kit; Wako Chemicals, Neuss, Germany) and triglyceride concentrations (L-type TG-H kit; Wako Chemicals) according to the manufacturer's protocols. The leptin concentrations of the medium also were 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; 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 medium 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 ($P < 0.05$), whereas 250 and 500 ng/ml leptin significantly decreased proliferation ($P < 0.01$, Fig. 1*A*). Similarly, 250 and 500 ng/ml leptin significantly decreased SV cell proliferation ($P < 0.01$, Fig. 1*B*). As expected, IGF-1 stimulated proliferation of both the adipose and SV fractions, by 49 and 23% respectively, compared with control (Fig. 1, *A* and *B*). AraC decreased preadipocyte and SV cell proliferation by 45 and 83%, respectively, compared with control (Fig. 1, *A* and *B*). Leptin had no effect on preadipocyte differentiation as measured by GPDH activity (data not shown), whereas 1 nM TNF-α decreased GPDH activity by 59% and 1,000 nM insulin increased it by 59% compared with control.

Medium 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 (Fig. 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 μg of 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*
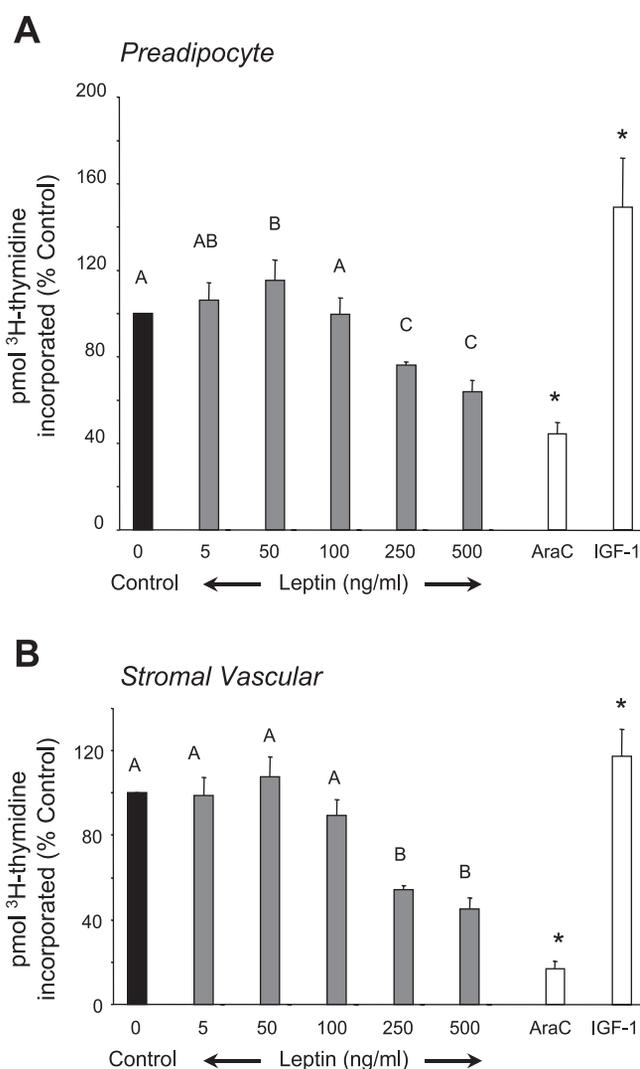

A *Preadipocyte*

B *Stromal Vascular*

Fig. 1. Proliferation of preadipocyte (*A*) and stromal vascular cells (*B*) treated with varying concentrations of leptin. Data represent means ± SE for 3–6 replicate cultures. [A,B,C]$P < 0.05$, significant effects of leptin on proliferation. Asterisks indicate a significant difference between positive (IGF-1) or negative (AraC, a DNA synthesis inhibitor) controls compared with the 0 ng/ml leptin condition.

and *5* of infusion compared with PBS-infused rats ($P < 0.01$, Table 1). The control rat serum, which was used in the test medium 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 (Fig. 3) but did not affect 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 medium from leptin- or PBS-infused rats, and there was no interaction between volume and source of medium on proliferation (adipose tissue-conditioned medium or control conditioned medium) (Table 2). In contrast, all concentrations of adipose tissue-conditioned medium significantly increased preadipocyte differentiation compared with conditioned medium not exposed to adipose tissue (Table 3). There
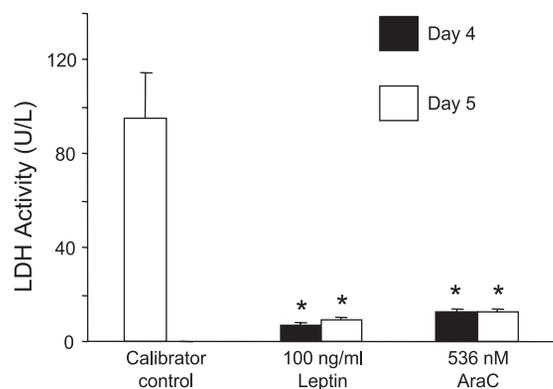
Fig. 2. Cell viability as measured by lactate dehydrogenase (LDH) activity in cell culture medium. Data are means ± SE for 4–6 replicates, each determined in quadruplicate. *$P < 0.05$, significantly lower LDH activity in medium collected from cells treated with 100 ng/ml leptin or AraC for 2 or 3 days compared with a lipid calibrator control provided by the manufacturer.

Fig. 3. Incorporation of [^{3}H]thymidine into the preadipocyte and stromal vascular cell fractions treated with 0.25% serum from rats infused with PBS or leptin. Data represent means ± SE for 6–10 rats, with each treatment determined in triplicate. *$P < 0.01$, significant difference between cells exposed to serum from PBS-infused and leptin-infused rats.

were no differences in the FFA or triglyceride concentrations of the conditioned medium from PBS- and leptin-infused rats (FFA: 0.10 ± 0.01 and 0.09 ± 0.01 ng/ml for PBS- and leptin-infused conditioned medium, respectively; triglycerides: 32 ± 5 and 31 ± 7 meq/l for PBS- and leptin-infused conditioned medium, 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 1,000 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 SV 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 medium 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 tissue-conditioned medium 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 given that the protein increases proliferation of other cell types, such as hematopoietic and endothelial cells (37). In addition, other investigators have reported that leptin

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

	PBS	Leptin
Start body weight, g	340±6	339±5
End body weight, g	344±6	336±5
Weight change, g/5 days	3.6±2.0	−2.8±2.4*
Food intake, g/day	19.5±2	18.0±1
Epididymal fat, g	2.7±0.1	2.9±0.3
Retroperitoneal fat, g	0.9±0.1	0.7±0.1
Perirenal fat, g	0.6±0.1	0.5±0.1
Mesenteric fat, g	1.5±0.1	1.0±0.2*
Serum leptin *day 3*, ng/ml	1.7±0.2	5.3±1.3*
Serum leptin *day 5*, ng/ml	1.2±0.2	3.2±0.6*

Values are means ± SE for 6–9 rats in the leptin group and 7–10 rats in the PBS group. *$P < 0.05$, significant difference between leptin- and PBS-infused rats.
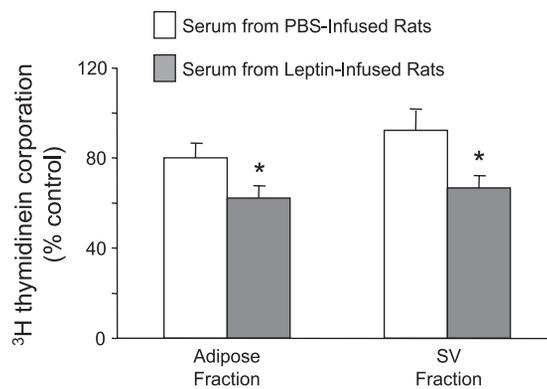
Table 2. *Incorporation of [^{3}H]thymidine into the preadipocyte and SV cell fractions in response to adipose tissue-conditioned medium and control conditioned medium*

Volume, %	Conditioned Medium Source		
	Control	PBS	Leptin
Preadipocyte fraction			
5	108±13	107±9	101±11
10	94±10	121±18	104±4
20	96±5	100±6	115±4
30	98±9	124±16	119±9
SV fraction			
5	89±8	94±97	87±7
10	86±24	113±14	104±6
20	100±14	107±11	121±9
30	85±9	109±13	132±9

Data are means ± SE ($n = 6$–7 rats), with each sample tested in triplicate, and represent [^{3}H]thymidine incorporated into preadipocyte and stromal vascular cell (SV) fractions, expressed as a percentage of control. There was no difference in preadipocyte proliferation within or between leptin- and PBS-infused groups. There was no interaction between volume of medium or type of medium (adipose tissue-conditioned medium or control conditioned medium).

Table 3. *GPDH activity as affected by varying concentrations of adipose tissue-conditioned medium and control conditioned medium*

	Conditioned Medium Source		
Volume, %	Control	PBS	Leptin
5	108±3*	141±12	144±14
10	103±6*	148±7	142±13
20	105±5*	150±11	153±15
30	96±5*	145±8	135±9

Data are means ± SE ($n = 6$–7 rats), with each sample tested in triplicate, and represent *sn*-glycerophosphate dehydrogenase (GPDH) activity, expressed as a percentage of control. There was no difference in differentiation within or between leptin- and PBS-infused groups. *$P < 0.05$, significant difference between adipose tissue-conditioned medium and control medium not exposed to adipose tissue.

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 in vitro was higher than the circulating concentrations found in normal animals. In young rats, physiological levels of circulating leptin are ~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 vs. 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 medium used in the proliferation assay provided an abundance of nutrients and contained high concentrations of insulin that are similar to baseline concentrations in conditions of obesity or to postingestive 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 medium. Thus, in conditions of positive energy balance in nonobese 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 nine times higher in obese than in normal individuals (14), which means that leptin in the extracellular fluid bathing adipocytes will 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 because 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 phosphatidylinositol 3-kinase (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 the possibility 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 medium from leptin-infused rats did not affect preadipocyte differentiation compared with serum or conditioned medium 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 medium from leptin-infused animals induced a change in preadipocyte differentiation, the response would have been detected. This negative result was somewhat surprising because leptin has been shown to reduce the size of mature adipocytes by inhibiting insulin-stimulated lipogenesis (5, 23) 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 by 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. Consis-

tent 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 experiment, 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 medium contained more leptin than the test medium 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 that 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 nonadipose tissues (58). TNF-α is produced by nonadipocyte cells in adipose tissue and is increased in tissue from obese individuals (17). Although TNF-α 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 TNF-α 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 (NK) cells, muscle protein accretion, and possibly direct reduction of adipose mass and is expressed mainly in skeletal muscle (1). 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 (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 α-melanocyte-stimulating hormone (α-MSH). Recent studies have shown significant weight loss in *ob/ob* mice treated with an α-MSH analog (18), and leptin administration doubles circulating concentrations of α-MSH in these mice (31). In vitro, α-MSH decreases adipocyte leptin expression and secretion (31). 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.

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 medium from leptin- or PBS-infused rats or with nonadipose control conditioned medium, 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 medium. This should have been, at least partially, accounted for by the 120-fold higher concentrations of conditioned medium tested (30% by volume) compared with the effective concentrations of serum (0.25% by volume). It also is possible that the 4-h exposure of medium 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 medium 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 medium; 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 medium.

In summary, the studies described 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 caused by 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.

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