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Lack of Effect of Leptin on Glucose Transport, Lipoprotein Lipase, and Insulin Action in Adipose and Muscle Cells*

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ABSTRACT

The effect of leptin on glucose transport, lipogenesis, and lipoprotein lipase activity was studied in cultured rat adipocytes and 3T3-L1 adipocytes. Leptin had no effect on basal and insulin stimulated glucose transport in isolated adipocytes from the rat and the genetically obese mouse. The incorporation of glucose into lipids was also unaffected. Lipoprotein lipase (LPL) activity remained unchanged in response to leptin in these cells, as well as in minced adipose tissue. Leptin also had no effect on both basal and insulin-stimulated glucose transport in cultured rat and human skeletal muscle cells. These studies showed that leptin had no effect on glucose transport, lipoprotein lipase activity, and insulin action in fat and muscle cells *in vitro*. (*Endocrinology* **139:** 2509–2513, 1998)

LEPTIN is the peptide product of the obese (*ob*) gene, and leptin secretion by adipocytes plays a key role in the regulation of energy metabolism and body weight. The genetically obese *ob/ob* mouse produces a defective leptin, resulting in massive obesity and hyperphagia (1). In addition to obesity, the *ob/ob* mouse has insulin resistant diabetes and defects in fat metabolism, thermogenesis, and reproduction. Whether the diabetes found in *ob/ob* mice is related to obesity alone, or to some other consequence of the defective leptin, is not known.

When *ob/ob* mice were treated ip with recombinant leptin, they not only ate less food and lost weight, but were also found to have decreased plasma insulin and glucose levels (2–4). It was also observed that very low doses of leptin were able to normalize blood glucose and body temperature without having any effect on food intake and body weight. Together with data demonstrating that the leptin receptor is found in many tissues, these results suggested that leptin may also have peripheral effects not related to satiety.

Several other reports have suggested that leptin has direct metabolic effects on cells. Emilsson *et al.* (5) have reported that leptin inhibited insulin secretion when added to culture of isolated pancreatic islet cells. In HEPG-2 cells, leptin inhibited insulin-stimulated insulin receptor substrate (IRS-1) tyrosine phosphorylation (6). In contrast to the inhibition of IRS-1 tyrosine phosphorylation, Takahashi *et al.* (7) have reported that leptin induced tyrosine phosphorylation of several proteins, including STAT-1, in renal adenocarcinoma cells. In cultured adipocytes, Bai *et al.* (8) demonstrated that leptin inhibited the insulin and dexamethasone-stimulated synthesis of fatty acids and total lipids. All these studies clearly indicate that leptin has direct effects on other metabolic pathways, in addition to its effect on the hypothalamus in regulating food intake.

In this report, we studied the direct effects of leptin on insulin action by examining glucose transport and lipoprotein lipase in differentiated preadipocytes from rat and 3T3-L1 cells, as well as in isolated adipocytes from the rat and the *ob/ob* mouse. We found no effect of leptin on insulin action, even in the *ob/ob* adipocytes, which would be expected to be very sensitive to exogenous leptin. Leptin also did not have any effect on the basal and insulin-stimulated glucose transport in rat and human skeletal muscle cells.

Materials and Methods

Chemicals and supplies

Recombinant leptin was obtained from Amgen (Thousand Oaks, CA) and Research Diagnostics, Inc. (Flanders, NJ). Both of these products were shown to be biologically active in two different obese mouse models, by the suppliers. Deoxyglucose, 3-isobutyl 1-methylxanthine, BSA, and dexamethasone were obtained from Sigma Chemicals (St. Louis, MO). 2-Deoxy (^{3}H)glucose and U-(^{14}C)-D-glucose were obtained from Dupont NEN (Boston, MA). Media, FBS, and all reagents for tissue culture were purchased from GIBCO (Grand Island, NY). Human recombinant insulin (Novolin) was obtained from Novo Nordisk (Princeton, NJ). All other reagents were of the best available grade.

Animals and adipocyte cultures

Male Sprague-Dawley rats, weighing between 180 and 220 g, were killed after an overnight fast, and the epididymal adipose tissue was

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removed immediately. Adipocytes were isolated from the adipose tissue by a collagenase digestion and were cultured for up to 24 h, as described previously (9). The same methods were used to obtain adipocytes from *ob/ob* mice, which were purchased from Jackson labs and were killed at 6–8 weeks of age.

Cell culture and differentiation

3T3-L1 cells were grown on 75-cm^2 culture flasks (Costar, Irvine, CA), in DMEM (Gibco BRL), supplemented with 10% FCS and an antibiotic mixture containing penicillin and streptomycin. For the experiments, cells were cultured in 12-well dishes, were grown to confluence, and were differentiated by incubation in DMEM medium with 10% FBS containing 1 μg/ml insulin, 0.5 mM isobutylmethylxanthine, and 0.25 μM dexamethasone for 48 h, according to the methods described by Clancy and Czech (10). Cells were then maintained in DMEM, containing 10% serum and 1 μg/ml insulin, for 3–4 days. Medium was then changed to DMEM, containing 10% FBS, for 2–3 days.

The stromal vascular fraction containing the preadipocytes was prepared from the collagenase digestion of the adipose tissue, as described previously (11), cultured in 12-well dishes, and differentiated using the methods described for 3T3-L1 cells.

The L6 cell culture was a gift from Dr. Amira Klip (The Hospital for Sick Children, Toronto, Ontario, Canada). These cells were maintained in α MEM, supplemented with 2% FBS, and an antibiotic mixture containing penicillin, streptomycin, and amphotericin, according to previous methods (12). For experiments, the cells were seeded in 12-well dishes and grown for 5–7 days, when they became differentiated into myotubes.

The procedures for culturing the muscle cells from human muscle biopsies and for the measurement of glucose transport were the same as those described by Ciaraldi *et al.* (13).

Glucose transport

3T3-L1 cells. The differentiated cells were treated with leptin (0.5 μg/ml) for 24 h before the day of the experiment. Before glucose transport assay, the cells were preincubated with 1 mL serum-free DMEM for 4 h. The cells were then washed twice with Krebs-Ringer phosphate buffer and incubated in the same buffer for 30 min. To determine insulin-stimulated glucose transport, insulin (10 nM) was added, and the incubation was continued for another 30 min. 2-Deoxy (^{3}H)glucose was added to produce a final concentration of 0.1 mM (1 μCi/100 nmol) and incubated for 10 min at room temperature. Leptin was added back to the respective wells during the transport assay. The medium was aspirated, and the cells were washed three times with PBS and dissolved in 0.5 ml of 0.2 N NaOH. Radioactivity in the lysate was determined by scintillation counting. Cell protein content was determined using BioRad reagent. Non-carrier-mediated transport of deoxyglucose was determined in the presence of 10 μM cytochalasin B.

Isolated mature adipocytes. Glucose transport in adipocytes, isolated from fat pads from rat and *ob/ob* mouse, was determined using the procedure described by Foley *et al.* (14). In this method, the uptake of (U-^{14}C)-D-glucose was measured at very low glucose concentration (300 nM). One ml of the cell suspension (5% lipocrit in DMEM) was incubated with leptin (0.5 μg/ml) for 1–24 h, as specified in the table or figure legends. At the end of this incubation, the cells were washed three times with PBS and incubated with 10 nM insulin for 60 min and then with 0.1 μCi of (^{14}C)-glucose (300 nM) for another 60 min at 37 C with shaking. Leptin was added back to the respective tubes of cells during these incubations. Then, 200 μl of the reaction mixture was transferred to a long microfuge tube containing 100 μl silicone oil. The tubes were then centrifuged for 20 sec, and top phase, containing the adipocytes, was cut and transferred to a scintillation vial to determine the radioactivity. Glucose transport was expressed as nanomoles of glucose.

L6 cells. Deoxy (^{3}H)glucose transport was assayed in these cells using the same method described for 3T3 cells, except that the concentration of deoxyglucose used was 10 μM.

Measurement of lipoprotein lipase (LPL)

LPL was measured, in the medium, after release with heparin, as described previously (15). The culture medium was removed from the dishes, and 1 ml of fresh medium containing heparin (10 u/ml) was added and incubated for 60 min at room temperature.

LPL catalytic activity was measured using an emulsified ^{3}H-triolein substrate, as described previously (16). After incubation of 100 μl of sample with 100 μl of substrate for 60 min, liberated ^{3}H-fatty acids were separated from the reaction mixture using the method of Belfrage (17). The LPL activity is expressed as nanomoles of fatty acids released in 60 min.

Glucose incorporation into lipids. Incorporation of ^{14}C-D-glucose into lipids was determined using the methods described by Lima *et al.* (9). Briefly, the adipocytes were washed and resuspended to 5% lipocrit. One ml of the cell suspension was incubated in a 20-ml plastic scintillation vial with leptin for 3 h and then with 0.1 μCi of (U-^{14}C)D-glucose (5 mM), with or without insulin (10 nM), for 1 or 2 h, as indicated, at 37 C with shaking. At the end of this incubation, the reaction mixture was treated with 5 ml Dole's reagent (isopropanol:n-heptane:H_2SO_4, 4:1:0.1, vol/vol) for lipid extraction (18).

Statistics. Data are presented as the mean ±SD. Student's *t* test was used to assess the significance of effects of leptin on the various parameters studied. $P < 0.05$ was the accepted level of significance.

Results

Glucose transport. To determine whether leptin affected glucose transport, 3T3-L1 cells were differentiated as described under *Materials and Methods* and were treated with leptin (0.5 μg/ml) for 24 h, after which, glucose transport was measured in the presence and absence of insulin. As shown in Fig. 1, insulin stimulated a 3- to 4-fold increase in glucose transport. In the cells treated with leptin, there was no effect on either basal or insulin-stimulated glucose transport.

Similar experiments were performed in primary cultures of rat adipocytes. Cells were prepared from a collagenase digestion of rat epididymal fat pads and were incubated in the presence or absence of leptin (0.5 μg/ml) for 1, 4, and 24 h. As shown in Fig. 2, leptin did not affect the basal or insulin-stimulated glucose transport in these cells.

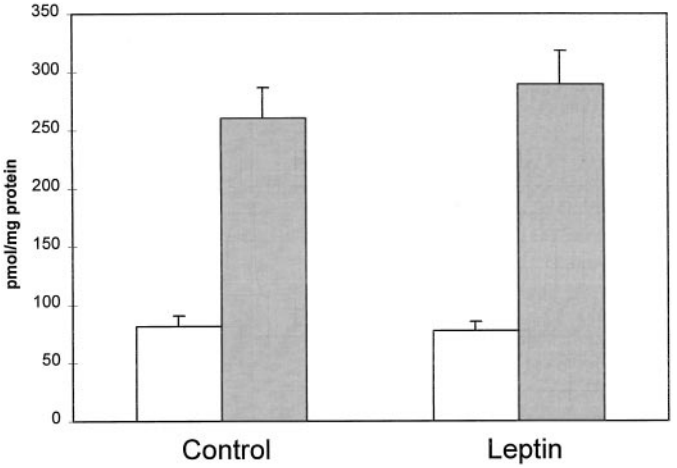

FIG. 1. Effect of leptin on 2-deoxyglucose transport in 3T3-L1 adipocytes. The cells were plated in 12-well dishes and allowed to differentiate using dexamethasone, isobutyl dimethylxanthine, and insulin. The differentiated cells were treated with leptin (0.5 μg/ml) for 24 h, and then the basal and insulin-stimulated transport of 2-deoxy(^{3}H)glucose were determined (□, basal; ■, insulin-stimulated). All the experimental details are as described in *Materials and Methods*. The values are expressed as picomoles of deoxyglucose per well. The protein concentration in the cells did not change after treatment with leptin. Each value is an average and SD from six determinations.

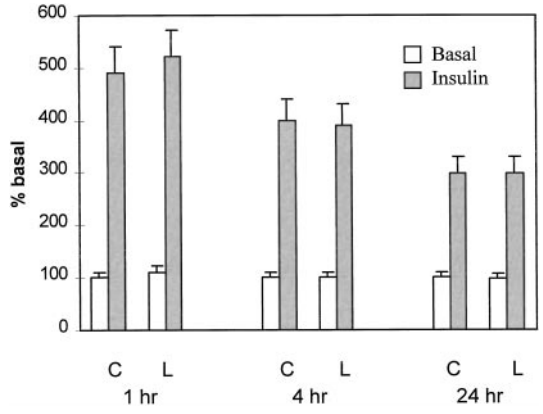

FIG. 2. Effect of leptin on (^{14}C)-D-glucose transport in rat adipocytes. Adipocytes were isolated from epididymal fat pads using collagenase digestion, then washed and resuspended in DMEM, to give a lipocrit of 5%. One milliliter of this suspension was incubated with or without leptin (0.5 μg/ml) for the indicated time period at 37 C in an incubator. At the end of this period, the cells were washed three times with PBS and incubated with 10 nM insulin for 60 min and then with 0.1 μCi of (^{14}C) glucose (300 nM) for another 60 min at 37 C with shaking. Leptin was added back to the respective tubes during this incubation. Further details of the procedure are described in the text. Each value is the mean and SD from triplicate determinations.

TABLE 1. Effect of leptin on glucose transport in *ob/ob* adipocytes

	Basal	Insulin
Control	4.9 ± 0.4	7.9 ± 1.1
Leptin	4.9 ± 0.7	6.3 ± 0.4
Control	2.3 ± 0.4	3.4 ± 0.2
Leptin 1	1.9 ± 0.1	2.8 ± 0.7
Leptin 2	2.3 ± 0.5	3.2 ± 0.1

Adipocytes were prepared from *ob/ob* fat pads and were treated with leptin (0.5 μg/ml) for 4 h. Leptin 1 and 2 were from different sources (see text). Data are expressed as pmol of glucose per assay.

Because *ob/ob* mice lack functional leptin, their adipocytes are likely to be more sensitive to the added leptin. We prepared isolated adipocytes from 6-week-old male *ob/ob* mice and studied the effect of leptin on glucose transport. As shown in Table 1, the addition of leptin had no significant effect on glucose transport in these cells. We used bioactive leptin from two different sources in this experiment.

An important site of insulin action is the muscle. To determine whether leptin altered insulin action in muscle cells, the L6 muscle cell line was used. Cells were cultured with 0.5 mg/ml leptin for 24 and 48 h, followed by the measurement of basal and insulin-stimulated glucose transport. As shown in Fig. 3, insulin showed a modest, though consistent, stimulation of glucose transport, but leptin had no effect on either basal or insulin-mediated transport. In addition to studying L6 cells, we examined the effects of leptin on glucose transport in human skeletal muscle cells from normal subjects, obese subjects, and patients with non-insulin-dependent diabetes mellitus. As shown in Table 2, there was no effect of leptin on either basal or insulin-stimulated glucose transport in cells from any of these subjects.

Glucose incorporation into lipid. Insulin stimulates glucose transport, as well as glucose metabolism. Although leptin had no effect on glucose transport, we decided to determine whether leptin had any effect on glucose metabolism, using the incorporation of [^{14}C]-glucose into total lipid in primary cultures of rat adipocytes. As shown in Table 3, insulin stimulated the incorporation of [^{14}C]-glucose into lipid. The addition of leptin had no effect, either on basal or insulin-stimulated lipid biosynthesis.

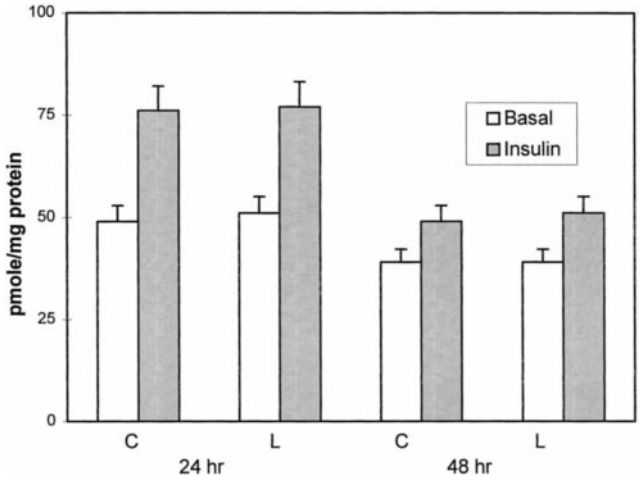

FIG. 3. Effect of leptin on 2-deoxyglucose transport in L6 muscle cells. The cells were cultured in 12-well dishes, and after differentiation, they were treated with leptin (0.5 μg/ml) for 24 or 48 h, as indicated. The uptake of 2-deoxyglucose was determined at the end of this period. All the experimental details are as described in *Materials and Methods*. The values are expressed as picomoles of 2-deoxyglucose per milligram of cell protein.

TABLE 2. Effect of leptin on glucose transport in cultured human skeletal muscle cells

		Basal	Insulin
Normal (n = 9)	Control	17.2 ± 4.3	21.8 ± 5.8
	Leptin	18.4 ± 4.6	20.9 ± 4.6
Obese (n = 4)	Control	14.3 ± 0.9	17.6 ± 1.4
	Leptin	15.5 ± 1.6	19.2 ± 2.1
NIDDM (n = 7)	Control	17.1 ± 4.2	22.5 ± 6.0
	Leptin	18.4 ± 4.6	21.6 ± 5.1

The methods for culturing the cells and glucose transport assay are described in *Materials and Methods.* The cells were treated with leptin (0.5 μg/ml) for 18 h. The numbers in parenthesis are the numbers of individuals studied. The values are pmol glucose/mg cell protein/min ± SD.

TABLE 3. Effect of leptin on the incorporation of ^{14}C-glucose into lipids in rat adipocytes

		nmol
Exp 1	Control	14.4 ± 1.6
	Leptin, 3 h	16.8 ± 2.0
Exp 2	Control, basal	24.4 ± 0.8
	Control, +insulin	36.8 ± 1.6
	Leptin (3 h) basal	23.2 ± 2.0
	Leptin, +insulin	40.4 ± 2.8

Freshly isolated rat adipocytes (1 ml of 5% lipocrit suspension) was used in each experiment. In exp 1, the cells were incubated with leptin (0.5 μg/ml) for 3 h and with ^{14}C-glucose for 1 h. In Exp 2, the cells were incubated with leptin for 3 h, insulin for 30 min, and subsequently with ^{14}C-glucose for 2 h. The experimental details are as described in the *Materials and Methods* section. The results are expressed as nmol of glucose incorporated into lipids. Means and SD from triplicate analyses are reported.

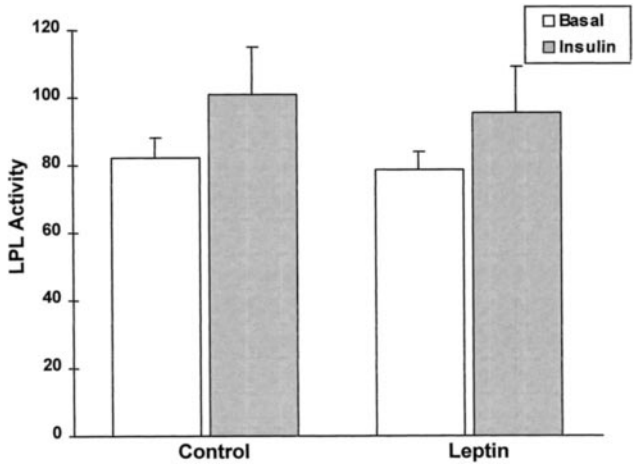

FIG. 4. Effect of leptin on LPL activity differentiated rat preadipocytes. The preadipocytes from rat adipose tissue were isolated, cultured in 12-well dishes, and allowed to differentiate using dexamethasone, isobutyl dimethylxanthine, and insulin, as described in *Materials and Methods*. The cells were then treated with leptin (0.5 μg/ml) for 24 h and 10 nM insulin overnight. Heparin-releasable LPL activity was determined, and the results are expressed as nanomoles of fatty acids released per milligram of cell protein. Each value represents the mean and SD from triplicate determinations.

TABLE 4. Effect of leptin on LPL activity in adipose tissue and isolated adipocytes from *ob/ob* mice

		LPL
Tissue	Control	38.1 ± 2.5
	Leptin, 3 μg/ml	34.5 ± 3.2
Adipocytes	Control	45.3 ± 0.2
	Leptin, 0.5 μg/ml	42.5 ± 0.6

Adipose tissue minces (0.5 g/ml DMEM) or adipocytes (2 ml of 5% suspension) were incubated with the indicated concentrations of leptin for 4 h at 37 C, and the heparin releasable LPL activity was determined. The LPL activity is expressed as nmol of fatty acids released per assay. The numbers given are mean and SD from triplicate determination.

Lipoprotein lipase activity. To determine whether leptin alters the basal or insulin-stimulated increase in LPL activity, rat preadipocytes were differentiated into adipocytes; and leptin, at a concentration of 0.5 μg/ml, was added to cultures for 24 h. As shown in Fig. 4, leptin had no effect on basal or insulin-stimulated LPL activity. Similar experiments were performed in *ob/ob* adipocytes, using both isolated adipocytes and adipose tissue pieces. Consistent with the insulin resistance of these cells, there was no effect of insulin on LPL activity. Nevertheless, the addition of leptin had no effect on LPL activity in these cells (Table 4).

Discussion

Ever since the identification and characterization of leptin and the leptin receptor, considerable research has been focused on the important central effects of leptin. However, because *ob/ob* mice are severely insulin resistant and diabetic, in addition to being obese, it is possible that leptin has peripheral effects, in addition to its central effects on appetite suppression. It has been suggested that the primary evolutionary role of leptin is to defend against starvation by rendering the animal infertile and stimulating the adrenocortical axis (19, 20). If the defense against starvation is the primary role of leptin, then leptin may continue to defend against possible future starvation by stimulating adipose tissue storage during the well-fed state. This could be accomplished through a peripheral role for leptin in stimulating glucose transport, glucose metabolism, and LPL in adipose tissue.

Several reports have suggested that leptin has direct effects on peripheral cells. Cohen *et al.* (6) found that leptin (48 ng/ml) inhibited insulin-stimulated IRS-1 tyrosine phosphorylation in HEPG-2 cells. This inhibitory effect would be expected to impair insulin action in liver, leading to elevated hepatic glucose output. In another study, Takahashi *et al.* (7) showed that leptin induced tyrosine phosphorylation of several proteins, including STAT-1, in renal adenocarcinoma cells. In adipocytes, Bai *et al.* (8) showed that leptin inhibited the insulin and dexamethasone-stimulated synthesis of fatty acids and total lipids. Such an inhibition of lipid synthesis would go against a role for leptin as an anabolic hormone. Emilsson *et al.* (5) described a leptin-mediated inhibition of insulin secretion by isolated pancreatic islet cells. Leptin also serves as the adipose-brain signal for the onset of sexual maturation (19, 20) and is part of the signal for the pulsatile release of gonadotropins. In a recent study, it was reported that leptin inhibited the FSH and IGF-I-stimulated progesterone production by rat ovarian granulosa cells (21). These studies provide evidence that leptin may have direct effects on other metabolic pathways, in addition to its effect on the hypothalamus in regulating food intake.

In this study, we searched for an effect of leptin on both basal and insulin-stimulated glucose or lipid metabolism in adipocytes and muscle cells, but found no effect. Because the cell lines, such as 3T3-L1 cells, are sometimes not representative of primary cultures, we examined glucose transport, [^{14}C]-incorporation into lipids, and LPL activity in rat adipocytes. In addition, we examined the effects of leptin on *ob/ob* cells. Because these cells are not exposed to leptin *in vivo*, they would likely be most sensitive to leptin, just as the *ob/ob* mouse is much more sensitive to the appetite-suppression effects of injected leptin. The lack of effect of leptin on insulin, glucose transport on LPL activity in adipocytes in our studies indicates that leptin-mediated partial reversal of diabetes is probably mediated through the hypothalamus, involving decreased food intake and stimulation of the autonomous nervous system. In different studies reported in the literature, the time required to detect the metabolic changes in response to leptin varied from 10 min to 2 days. In our studies, we have incubated the cells with leptin for up to 48 h, wherever possible. In the case of isolated adipocytes, we used 3–24 h incubation because it was difficult to keep them functionally intact beyond 24 h.

A recent study described a leptin-mediated inhibition of insulin action in adipocytes (22). In another recent report, leptin was shown to increase basal glucose transport and glycogen synthesis in C_2C_{12} muscle cells (23). In both these studies, the source of leptin was different from that used by us, although it is not clear why this should be important, given that both preparations are recombinant and pure and have been tested for bioactivity. In the study by Müller *et al.* (22), adipocytes were cultured with leptin in a medium containing high glucose (25 mM) and adenosine, an insulin ag-

onist, and this may have affected the data. In another recent study, Mick *et al.* (24) have observed that leptin has no effect on glucose transport and insulin action in isolated rat adipocytes.

Leptin receptors exist in two major forms, the long one with the complete intracellular domain and the short one with truncated intracellular domain. The long form is thought to mediate the biological effects of leptin and is expressed in large amounts in the choroid plexus and the hypothalamus and, to a smaller extent, in lung and kidney. Using PCR, Lee *et al.* (25) demonstrated the presence of several forms of the leptin receptor in adipose tissue. Although the short form of the leptin receptor is predominant (26) in the adipose tissue, detectable levels of the long functional form have been reported in one study (25) but not in another (5). Yamashita *et al.* (27) have recently reported that the short form of the leptin receptor can also perform signal transduction, when expressed in Chinese hamster ovary cells, although (to a lesser extent, compared with the long form). The physiological significance of this finding in these genetically altered cells needs to be determined. Therefore, it is not clear whether adipocytes have adequate levels of functional leptin receptors to interact with leptin and to be metabolically regulated.

Although Ghilardi *et al.* (26) have shown that skeletal muscle does not express the functional form of leptin receptor, Liu *et al.* (28) have reported that leptin inhibited glycogen synthesis in soleus muscle from *ob/ob* mice *in vitro*. On the contrary, Berti *et al.* (23) have shown that leptin increased glycogen synthesis in cultured muscle cells. Muoio *et al.* (29) have found that leptin stimulated fatty acid oxidation and inhibited triglyceride synthesis in isolated muscle preparation. Therefore, the peripheral effect of leptin on the metabolism in muscle is uncertain.

In summary, we examined the effects of leptin on glucose transport, glucose metabolism, and LPL activity in adipocytes and cultured muscle cells, and we found no effect. These data suggest that the predominant effects of leptin are mediated through its action on the hypothalamus and not through direct effects on muscle or adipose tissue.

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