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Lipolytic Effect of *in Vivo* Leptin Administration on Adipocytes of Lean and *ob*/*ob* Mice, but Not *db*/*db* Mice

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The present study has examined the effect of a single *in vivo* intraperitoneal injection of the adipocyte-derived hormone, leptin, on the *in vitro* lipolysis of fat cells of different types of mice. Administration of 1 and 10 mg leptin per kg body weight to *ob*/*ob* mice significantly increased ($P < 0.0001$) the basal lipolytic activity compared to *ob*/*ob* mice receiving vehicle solution (phosphate-buffered saline, PBS). The highest leptin dose tested (10 mg/kg body weight) produced a threefold increase in basal lipolysis. In lean mice administration of 10 mg leptin per kilogram of body weight produced an increase in basal lipolysis of 52.7% ($P < 0.01$). However, in *db*/*db* mice none of the three leptin doses injected had a significant effect on the lipolytic activity of adipocytes relative to basal lipolysis observed in *db*/*db* mice injected with PBS only. These data provide evidence for a lipolytic effect of leptin on white adipose tissue, which operates independently from changes in food intake, body weight, and the size of the fat stores.

The cloning in late 1994 of the *ob* gene led to the identification of a 167-amino acid cytokine-like peptide, termed OB protein or leptin, synthesized by adipocytes and secreted into the bloodstream (1, 2). Subsequent physiological studies revealed that administration of leptin to *ob*/*ob* mice lowered their body weight, percent body fat and food intake as well as enhanced their energy expenditure (3–5). These effects strongly implicated leptin as a negative feedback signal, which reflects body adiposity by acting at the hypothalamic level (3–5).

Although the functional leptin receptor (OB-Rb) has been shown to be highly expressed in the hypothalamus, OB-Rb mRNA has been also demonstrated in other organs, including pancreas, kidneys, adrenals, ovaries and white adipose tissue among others (6–9). Recently, direct physiological effects of leptin on some of these non-hypothalamic target organs, such as on hematopoietic cells, the endocrine pancreas, and the ovaries have been described (8, 10–12). Previous studies have shown an *in vitro* lipolytic effect of leptin on white adipose tissue, thus providing evidence for a possible autocrine/paracrine role of leptin (13, 14). However, *in vitro* studies may not be representative of *in vivo* responses due to the absence of appropriate feedback systems, compensatory mechanisms and secondary responses. Therefore, the aim of the present study was to test whether *in vivo* leptin administration was capable of increasing the basal lipolytic activity of white adipocytes. A single leptin injection was used to avoid potential confounding changes resulting from hypophagia and body weight loss following long-term leptin administration.

METHODS

Materials. Recombinant murine leptin was purchased from PeproTech EC Ltd. (London, UK). The OB protein showed more than 95% purity as evidenced by SDS–PAGE and HPLC analyses. Leptin was dissolved in phosphate-buffered saline (PBS) at pH 7.4. Bovine serum albumin (fraction V) was purchased from Sigma Chemical Co. (St. Louis, Missouri). Collagenase P (from *Clostridium histolyticum,* activity 1.52 U/mg) as well as glycerol-3-phosphate dehydrogenase and glycerolkinase were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). All other chemicals and organic solvents were of reagent grade.

Animals. All experimental procedures were conducted according to institutional guidelines for Animal Care and Use at the University of Navarra. Eight-week-old female C57BL/6-*ob*/Ola/Hsd ($n = 40$) and C57BL/6-*db*/Ola/Hsd mice ($n = 40$) weighing 43 ± 2 g were obtained from Harland (UK) and are referred to as *ob*/*ob* and *db*/*db* mice, respectively. Lean (+/?) littermates ($n = 40$) weighing 25.1 ± 0.4 g were also obtained from Harland (UK). To evaluate the dose-response effect of leptin on the lipolytic activity of white adipose tissue, animals were fasted overnight with free access to water and were randomly divided into a control and three leptin-treated groups containing ten mice each. Animals were injected intraperitoneally (ip) with equivolume injections (10 ml/kg body weight) of either PBS in the control group or OB protein in the three leptin-treated groups. The leptin doses tested were 0.1, 1.0 and 10.0 mg/kg body weight. Mice were sacrificed by decapitation exactly 1 h after ip injection.

Adipocyte isolation and lipolysis measurements. Abdominal fat depots located on the opposite part to the ip injection site were immediately dissected out and weighed. Each of the obese animals provided enough adipose tissue to be considered as an individual sample.

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In the lean group, it was necessary to pool the fat pads of two mice in order to obtain a single sample. Adipocyte isolation was performed following the method of Rodbell (15) as modified by Galitzky *et al.* (16) and as described earlier (13). In brief, white adipose tissue samples were cut into small pieces and were digested at 37°C with collagenase P in Krebs-Ringer bicarbonate buffer containing albumin (3.5 g/100 ml) and glucose (6 mM) at pH 7.4 [KRBA]. The ratio of digestion solution to adipose tissue mass was 5 ml/g and 0.75 ml of KRBA per mg of collagenase. After 90 min of incubation under continuous vigorous shaking (90 cycles/min) the fat cells were filtered through nylon mesh and washed three times with KRBA to eliminate the stromavascular fraction and collagenase. The fat cells were brought to a suitable dilution in KRBA buffer. Adipocytes were incubated in polyethylene tubes with continuous gentle shaking (30 cycles/min) in a water bath at 37°C. After 90 min, the incubation tubes were placed in an ice bath and 200 μl of the infranatant were removed for enzymatic determination of glycerol released into the incubation medium, which was taken as the index of lipolytic rate (17). Total lipid content was evaluated gravimetrically after extraction as described by Dole and Meinertz (18). Data are expressed as μmol of glycerol released per 100 mg of total lipids after a 90 min incubation in KRBA.

Statistical analysis. All results are given as means ± SEM. The effect of mouse strain and leptin dose on lipolysis were evaluated by two-way repeated measures analyses of variance (ANOVA). Intergroup differences in the effects to each leptin concentration were further computed by one-way ANOVA followed by Fisher's protected least significant difference (PLSD) *post hoc* pairwise comparison. Statistical significance between the different leptin concentrations tested were confirmed by unpaired two-tailed *t* tests. Values were considered to be statistically significant when $P < 0.05$. Analyses were performed using the StatView 4.01 Non-FPU (Abacus Concepts Inc., Berkeley, CA) statistical package for Apple Macintosh computers.

RESULTS

Injection of leptin resulted in a dose-dependent increase in basal lipolysis of *ob*/*ob* mice. Two-way repeated measures ANOVA revealed a significant overall effect of leptin dose ($F = 20.62$; $P < 0.0001$) and mouse strain ($F = 78.83$; $P < 0.0001$), along with a significant dose x strain interaction ($F = 20.11$; $P < 0.0001$). Adipocytes obtained from all *ob*/*ob* mice ip injected with leptin showed an increase in the average baseline lipolytic activity values compared to animals injected with PBS. As illustrated in Fig. 1, administration of 1.0 and 10.0 mg leptin per kg body weight increased the basal lipolytic activity two-fold and almost three-fold, respectively. Although the lowest leptin dose administered (0.1 mg/kg body weight) produced a 16.1% increase in basal lipolysis, no statistically significant differences were found compared to values obtained in mice which received PBS injections. All other pairwise comparisons performed showed statistically significant differences ($P < 0.001$) in the glycerol released by isolated fat cells of *ob*/*ob* mice.

The basal lipolytic activity was significantly increased ($P < 0.001$) in lean mice that received 10 mg leptin per kg body weight compared to animals injected with PBS. Although lower doses of leptin (0.1 and 1.0 mg/kg body weight) increased lipolysis approximately 19%, the effect did not reach statistical significance.

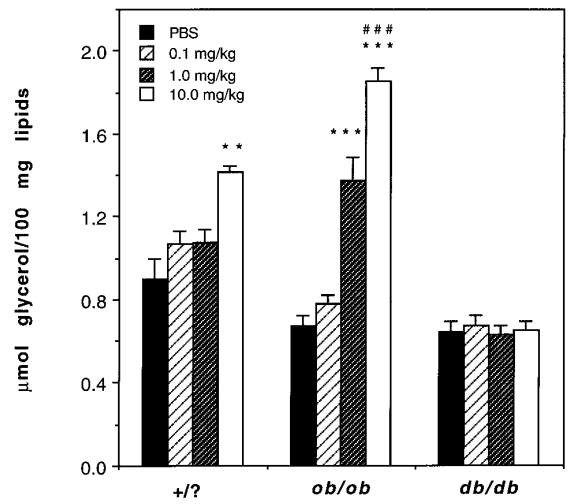

FIG. 1. Effects of intraperitoneal (ip) leptin injection on basal lipolysis of adipocytes isolated from lean (+/?), *ob*/*ob*, and *db*/*db* mice 1 h after OB protein administration (0.1, 1.0, and 10.0 mg leptin/kg body weight). Values represent means ± SEM ($n = 10$/group for both *ob*/*ob* and *db*/*db* mice; $n = 5$/group for lean littermates) after 90 min of incubation. $^{**}P < 0.001$ vs +/? animals receiving ip injections of phosphate-buffered saline (PBS), 0.1 or 1.0 mg leptin/kg body weight; $^{***}P < 0.0002$ vs *ob*/*ob* animals receiving ip injections of either PBS or 0.1 mg leptin/kg body weight; $^{###}P < 0.003$ vs *ob*/*ob* mice injected with 1.0 mg leptin/kg body weight.

In contrast to lean and *ob*/*ob* mice, in *db*/*db* mice, i.e., the mutants that lack functional OB-R, injection of leptin, even at the highest dose, had no statistically significant effect on the lipolytic activity of adipocytes relative to basal lipolysis values obtained in mice receiving PBS only.

DISCUSSION

The present study shows that a single ip leptin injection stimulates lipolysis in adipocytes of lean and *ob*/*ob* mice. The observation that already 1 h after administration leptin increases the basal lipolytic activity indicates that some metabolic and hormonal actions of leptin precede its effects on appetite and body weight suggesting that this effect is of physiological relevance and not a mere consequence of food intake or body weight changes per se. In this context, it is interesting to consider the study of Harris *et al.* (19), which tried to separate physiological from potentially pharmacological responses of OB protein administration by using a wide range of leptin concentrations. In *ob*/*ob* mice doses as low as 2 μg leptin per day for 7 days were shown to reduce the weight of all white fat pads measured, i.e., retroperitoneal, inguinal, perirenal, mesenteric and gonadal fat depots, strongly supporting a physiological rather than a pharmacological effect of leptin on white adipose tissue (19).

Our findings are also consistent with pair-feeding

experiments supporting the hypothesis that leptin exerts adipose-reducing effects beyond what can be accounted for by a reduction in food intake (20). Furthermore, the increased rate of lipolysis observed in white fat pads in our experiments is in agreement with other studies reporting that *in vivo* administration of leptin dramatically reduces tissue triglyceride stores compared with pair-fed controls (21). In addition, the same group has demonstrated an increased expression of lipolytic enzymes in primary cultures of pancreatic islets (22), which is compatible with our findings in white adipose tissue.

The differences observed in the leptin-induced lipolysis in *ob*/*ob* mice relative to their lean littermates indicates that *ob*/*ob* mutants are especially sensitive to the effects of the OB protein. This observation is consistent with previous results showing a diminished effect of leptin on food intake, body weight and *in vitro* lipolysis on wild-type mice relative to *ob*/*ob* mice (3–5, 13, 19). In this sense, the quantitative differences between lean and *ob*/*ob* animals may reflect a substantial upregulation of OB-R activity in the *ob*/*ob* mouse in the absence of functional circulating leptin.

Pretreatment with leptin, even at the highest OB protein concentration tested, did not stimulate the lipolytic rate of adipocytes obtained from *db*/*db* mice. The absence of a leptin-induced lipolytic effect in these mutants has been also observed in previous *in vitro* studies (13). The lack of effect of leptin on these animals may rest with the fact that the *db* mutation resides in the gene encoding the OB-Rb (7, 23). Pharmacologically, the further administration of leptin could be considered to overcome leptin resistance, as has been shown in obese *fa*/*fa* rats. However, in that experiment, leptin was supplied as an intracerebroventricular bolus injection (24).

The present experiment does not indicate whether the leptin-induced lipolysis observed is due to a direct effect of leptin on adipocytes or secondary to an interaction with hypothalamic receptors. Several independent lines of evidence support that leptin has direct effects on white adipose tissue at a metabolic and molecular level: (i) leptin at concentrations as low as 0.01 nM stimulates *in vitro* lipolysis of white adipocytes (13); (ii) leptin increases the baseline lipolytic activity in a time- and dose-dependent manner (13, 14), effects which are absent in fat pads from *db*/*db* mice (13) and *fa*/*fa* rats (14); (iii) *in vitro* and *in vivo* treatment of adipocytes with leptin induces the nuclear translocation of specific signal transducers and activators of transcription implicated in the signaling pathway of OB-Rb (14); (iv) OB-R mediates apparent autocrine regulation of leptin gene expression (25). However, the possibility of a paracrine as well as a centrally-mediated effect working together may not be ruled out. Recently Qian *et al.* (26) have shown that intracerebroventricular administration of leptin causes deletion of adipocytes by apoptosis. This finding raises the possibility that leptin treatment induces the deletion of adipocytes in addition to increasing lipolysis. Moreover, it is likely that other hormones and the sympathetic nervous system are partly responsible for the leptin-induced lipolysis observed. In this sense, glucocorticoids and insulin have been found to stimulate *ob* gene expression both *in vivo* and *in vitro,* and insulin in particular has been proposed to exert a key role in leptin regulation (27–30). In contrast, administration of triiodothyronine to hypothyroid rats results in a decrease in *ob* mRNA in white adipose tissue associated with a parallel decline in circulating leptin levels (31). Finally, catecholamines have been reported to inhibit *ob* gene expression and reduce serum leptin concentrations primarily through β_3-adrenoceptors (32–34).

In summary, our findings provide evidence for a lipolytic effect of leptin on white adipose tissue, which operates independently from changes in food intake, body weight and fat size stores.

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