Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1726–1730, February 1996
Physiology

Decreased food intake does not completely account for adiposity reduction after ob protein infusion

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Communicated by David Botstein, Stanford University School of Medicine, Stanford, CA, November 20, 1995 (received for review September 1, 1995)

ABSTRACT **The effects of recombinantly produced ob protein were compared to those of food restriction in normal lean and genetically obese mice. Ob protein infusion into *ob/ob* mice resulted in large decreases in body and fat-depot weight and food intake that persisted throughout the study. Smaller decreases in body and fat-depot weights were observed in vehicle-treated *ob/ob* mice that were fed the same amount of food as that consumed by ob protein-treated *ob/ob* mice (pair feeding). In lean mice, ob protein infusion significantly decreased body and fat-depot weights, while decreasing food intake to a much lesser extent than in *ob/ob* mice. Pair feeding of lean vehicle-treated mice to the intake of ob protein-treated mice did not reduce body fat-depot weights. The potent weight-, adipose-, and appetite-reducing effects exerted by the ob protein in ob protein-deficient mice (*ob/ob*) confirm hypotheses generated from early parabiotic studies that suggested the existence of a circulating satiety factor of adipose origin. Pair-feeding studies provide compelling evidence that the ob protein exerts adipose-reducing effects in excess of those induced by reductions in food intake.**

Recent epidemiologic studies have reported that more than one-third of U.S. adults 20 yr of age or older are overweight and that this prevalence increased by 8% over a 15-yr period (1). Obesity is associated with increased risk for several co-morbid conditions and diseases, including insulin resistance, non-insulin-dependent diabetes mellitus, cardiovascular disease, hypertension, hypertriglyceridemia, dyslipoproteinemia, and some forms of cancer (2, 3). The recent cloning and sequencing of the mouse *ob* gene and its human homologue (4) represent a significant step toward a better understanding of a possible biochemical cause of obesity.

Parabiosis experiments performed >20 yr ago predicted that the genetically obese (*ob/ob*) mouse does not produce a satiety factor that regulates its food intake, whereas the diabetic (*db/db*) mouse produces, but does not respond to, a satiety factor (5, 6). Recent reports have demonstrated that daily injections of recombinant ob protein profoundly inhibit food intake and reduce body weight and fat in *ob/ob* but not in *db/db* mice (7–9), suggesting that the ob protein is such a satiety factor, as proposed in early cross-circulation studies. Although modest effects of daily injections of the ob protein on food intake and body weight were reported in lean mice, there was a significant reduction in body fat as assessed by carcass composition in one (8) but not in another (7) of these reports, despite equivalent decreases in body weight. To elucidate the activity of the ob protein, a comprehensive analysis of the effects of low-dose continuous infusions of the ob protein in lean and obese mice on body weight, food intake, and adipose-depot mass is presented here, including a comparison of the effects of ob protein treatment to those of pair feeding in lean and obese mice. The data suggest that a significant biological role for the ob protein is that of metabolic regulation, in addition to appetite suppression.

MATERIALS AND METHODS

Protein Production. Murine ob cDNA was obtained by PCR from an adipocyte cDNA library using primers based on ref. 4. Mature ob protein (amino acids 22–167) was expressed in *Escherichia coli* by inserting the ob coding sequence in frame with the secretion sequence of the *E. coli* heat-stable enterotoxin II, downstream of the *E. coli* alkaline phosphatase promoter (10). After cell lysis, the insoluble fraction was solubilized in 8 M urea buffer, pH 8.35/25 mM dithiothreitol. Reduced ob protein was purified by size exclusion and reversed-phase HPLC, then refolded in the presence of glutathione. Refolded ob protein was purified by reversed-phase HPLC and analyzed by SDS/PAGE and amino acid and mass spectrometry analyses.

Animal Studies. All manipulations involving animals were reviewed and approved by Genentech's Institutional Animal Care and Use Committee. For *ad libitum* feeding studies, 7-week-old genetically obese C57BL/6J-*ob/ob* (*ob/ob*) and C57BL/KsJ-*db/db* (*db/db*) mice and lean littermates (heterozygous C57BL/6J-+/*ob* for *ob/ob* and wild-type C57BL/KsJ-+/m for *db/db*) were purchased from The Jackson Laboratory. The genotype of the C57BL/6J-+/*ob* mice was confirmed by PCR amplification followed by restriction endonuclease digestion of a 150-bp segment of DNA that encompasses the region of the point mutation responsible for the *ob* phenotype (4). The *ob/ob* mutation results in an additional cleavage site for the restriction endonuclease *Dde* I in the mutant allele. Mice were housed in groups of four or five with *ad libitum* access to water and standard mouse chow (Purina 5010) in a temperature-, humidity-, and light-controlled (lights on at 06:00 hr, off at 18:00 hr) colony room.

Miniosmotic pumps (Alzet model 2002; Alza) were filled with purified recombinant ob protein (100 μg/kg per day) in sterile phosphate-buffered saline (PBS) or PBS alone under sterile conditions following manufacturer's instructions and incubated overnight in sterile saline at room temperature before implantation into mice. Mice were anesthetized with ketamine/xylazine, and miniosmotic pumps were implanted s.c. in the midscapular region. The body weight of each mouse (to the nearest 0.1 g) and the weight of the food contained in the food bin in each cage (to the nearest 0.1 g) were recorded between 17:00 hr and 18:00 hr every 1 to 2 days. Mice were killed by barbiturate overdose followed by exsanguination via cardiac puncture.

Fat pads and organs were immediately dissected, blotted, and weighed to the nearest 0.001 g. Hepatic glycogen content was assessed on paraffin-embedded liver sections that were fixed in 10% neutral-buffered formalin and stained by the periodic acid Schiff reaction with or without previous diastase

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digestion. Hepatic lipid content was assessed on fresh frozen liver sections that were stained with Oil Red O. Fat pads were histologically examined after fixation in 10% neutral-buffered formalin, sectioning, and hematoxylin/eosin staining. Blood samples (≈0.2 ml) were obtained from the retroorbital sinus of conscious mice on day 13 of treatment at 14:00 hr, after a 5-hr fast. Blood was stored on ice until centrifugation, and then serum was stored at −20 C° until use. Serum insulin concentrations were determined by radioimmunoassay (Linco Research, St. Louis). Serum concentrations of glucose, cholesterol, and triglycerides were determined on a Technicon Chem1 + System chemistry analyzer (Bayer, Tarrytown, NY).

For pair-feeding studies, 8-week-old obese (C57BL/6J-*ob*/*ob*; The Jackson Laboratory) or lean (C57BL/6; Charles River Breeding Laboratories) female mice were housed as described above. The three treatment groups for each genotype were *ad libitum*-fed PBS-treated, *ad libitum*-fed ob protein-treated, or pair-fed PBS-treated. The ob protein was delivered via miniosmotic pumps as described above at a dose of 270 μg/kg per day. Pair feeding was accomplished by measuring the food intake of the *ad libitum*-fed ob protein-treated mice every 24 hr and presenting this amount of food to the pair-fed PBS-treated mice. For each of the three treatment groups there were two to three cages of mice, containing two to five mice per cage. Blood samples were obtained, the mice were killed, and tissues were harvested as described above.

Data Analysis. All data are presented as the mean ± SEM and were analyzed by ANOVA with *post hoc* differences determined by Fisher's protected least significant difference test if ANOVA was significant at the level of $P < 0.05$.

RESULTS

***Ad Libitum* Feeding Studies.** Infusion of the ob protein markedly decreased body weight (Fig. 1*A*) and food intake (Fig. 1*B*) in *ob*/*ob* mice throughout the treatment period. The absolute (mg) and relative weights (mg of fat pad/g of body wt) of two major white adipose depots, the retroperitoneal and inguinal fat pads, were significantly smaller in *ob*/*ob* mice as a consequence of ob protein infusion (Table 1). Histologic examination of these fat pads did not reveal any evidence of fat cell atrophy with ob treatment in *ob*/*ob* mice. Both the absolute and the relative liver weights were significantly lower in the ob protein-treated *ob*/*ob* mice (Table 1). Histologically, livers from *ob*/*ob* mice treated with ob protein were depleted of their glycogen content, whereas hepatic lipid storage was mildly decreased (data not shown).

ob/*ob* mice were hyperglycemic, hyperinsulinemic, and hypercholesterolemic compared to lean controls (Table 1). Fasting serum concentrations of glucose, insulin, and cholesterol were lowered by ob protein infusion in *ob*/*ob* mice to levels similar to those observed in their lean genetic controls (+/*ob*; Table 1). Infusion of the ob protein significantly decreased absolute kidney (24%) and heart (12%) weights; however, when expressed relative to body weight only the change in kidney weight retained statistical significance. No statistically significant effects of ob protein treatment were observed on the absolute weights of the spleen, adrenal glands, or gastrocnemius muscle of *ob*/*ob* mice.

The increase in body weight in *db*/*db* mice treated with ob protein (7.8% of initial body weight) was not significantly different from that of PBS-treated *db*/*db* mice (9.7% of initial body weight; Fig. 1*A*). No large decreases in food intake occurred in *db*/*db* mice, such as were observed in *ob*/*ob* mice, in response to ob protein treatment (Fig. 1*B*). Infusion of the ob protein into *db*/*db* mice did not affect either the absolute or the relative weights of any of the organs examined (Table 1; also spleen, kidney, heart, thymus, adrenal glands, and gastrocnemius muscle, data not shown). There were no histologic changes due to ob protein treatment in liver or fat-pad specimens from *db*/*db* mice. *db*/*db* mice were hyperglycemic, hyperinsulinemic, and hypercholesterolemic compared to their lean genetic controls (+/m; Table 1), and infusion of the ob protein into *db*/*db* mice did not affect these end points (Table 1).

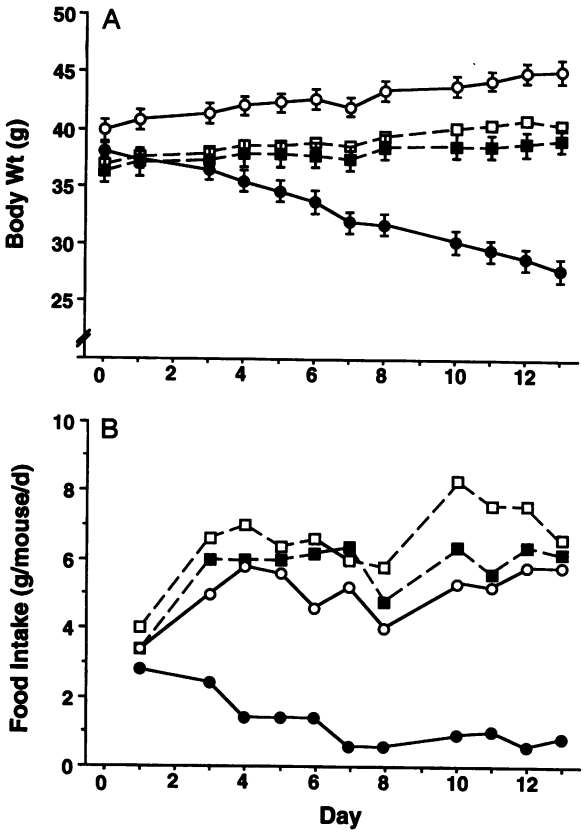

FIG. 1. Body weights (*A*) and food intake (*B*) of female obese mice (circles connected with solid lines, C57BL/6J-*ob*/*ob*; squares connected with dashed lines, C57BL/KsJ-*db*/*db*) treated with ob protein (100 μg/kg per day, solid symbols) or PBS (open symbols) delivered continuously via miniosmotic pumps implanted s.c. on day 0. Body-weight data are the mean ± SEM. $n = 5$ per group. Food-intake data are the number of grams consumed per cage per day divided by the number of mice in the cage.

Infusions of the ob protein into the lean littermates of the genetically obese mice resulted in significant reductions in body-weight gain accompanied by significantly smaller adipose depots (Fig. 2*A* and Table 1). Modest, transient decreases in food intake were seen in lean mice treated with ob protein (Fig. 2*B*), in contrast to the large and persistent reductions seen in genetically obese *ob*/*ob* mice treated with ob protein (Fig. 1*B*). In both lean genotypes, ob protein treatment resulted in moderate to severe fat-cell atrophy characterized by diffuse shrinkage of adipocytes, often with multiple microvacuoles of fat and occasionally an absence of fat vacuoles in severely affected regions (data not shown). Statistically significant decreases in both the absolute and relative liver weights were observed after ob protein treatment in +/*ob* but not in +/*m* mice (Table 1). Histologically, hepatic lipid and glycogen content were within normal limits after ob treatment in both lean genotypes. The absolute weights of the remainder of the organs surveyed (spleen, kidneys, heart, thymus, adrenal glands, and gastrocnemius muscle) were not affected by treatment with ob protein. No significant decreases in fasting serum levels of glucose, insulin, cholesterol, or triglycerides were observed after *ob* infusion into the lean genetic controls (Table 1). Similarly conducted ob protein infusion studies in lean normal C57BL/6 mice from another commercial vendor (Charles River Breeding Laboratories) yielded identical re-

Table 1. Effects of ob protein infusion (100 μg/kg per day s.c. for 14 days) on change in body and organ weights and fasting serum end points in 7-week-old genetically obese mice and lean littermates

	Genotype							
	ob/ob		*db/db*		*+/ob*		*+/m*	
Treatment	PBS	ob	PBS	ob	PBS	ob	PBS	ob
Change in body wt*	5.3 ± 0.4	−10.2 ± 0.5‡	3.6 ± 0.3	2.9 ± 0.4	1.2 ± 0.3	−0.6 ± 0.2‡	2.0 ± 0.4	0.8 ± 0.1‡
Absolute RP wt†	658 ± 60	323 ± 32‡	372 ± 42	452 ± 36	78 ± 8	12 ± 3	25 ± 3	4 ± 0.7
Relative RP wt†	14.5 ± 1.0	11.5 ± 0.9‡	9.2 ± 1.0	11.6 ± 1.1	3.6 ± 0.4	0.65 ± 0.2‡	1.3 ± 0.1	0.2 ± 0.04‡
Absolute ING wt†	4142 ± 203	2132 ± 194‡	3662 ± 98	3603 ± 46	476 ± 36	154 ± 23	315 ± 14	111 ± 9
Relative ING wt†	91.3 ± 2.9	76.2 ± 5.3‡	90.4 ± 3.2	91.9 ± 2.2	22.3 ± 2.0	8.1 ± 1.2‡	16.3 ± 0.9	6.3 ± 0.5‡
Absolute liver wt†	2770 ± 113	937 ± 44‡	2407 ± 90	2190 ± 92‡	1061 ± 84	839 ± 20‡	1051 ± 11	931 ± 11
Relative liver wt†	61.1 ± 1.5	33.6 ± 1.1‡	59.3 ± 1.7	55.7 ± 1.5	49.6 ± 4.4	44.3 ± 1.1	54.4 ± 0.5	52.9 ± 1.1
Glucose, mg/dl	229 ± 21	141 ± 5‡	434 ± 43	482 ± 31	154 ± 4	149 ± 3	135 ± 8	136 ± 8
Insulin, ng/ml	12.5 ± 0.8	9.1 ± 0.3‡	14.3 ± 1.2	13.9 ± 1.6	8.0 ± 0.3	7.9 ± 0.2	8.8 ± 0.4	9.0 ± 0.3
Cholesterol, mg/dl	138 ± 26	58 ± 6‡	150 ± 6	132 ± 4	74 ± 2	71 ± 4	72 ± 8	60 ± 2
Triglycerides, mg/dl	78 ± 11	59 ± 4	171 ± 10	155 ± 25	68 ± 6	55 ± 3	66 ± 4	71 ± 15

RP, retroperitoneal fat pads; ING, inguinal fat pads. All data are the mean ± SEM. $n = 5$ per group except $+/m$ treated with PBS, where $n = 4$.
*Grams gained or lost from day 0–14.
†Absolute organ weights expressed as mg; relative organ weights expressed as mg/g of body weight.
‡$P < 0.05$ versus PBS-treated control within genotype.

sults with respect to all end points discussed above (data not shown).

Pair-Feeding Studies. Ob protein-induced body-weight loss in *ob/ob* mice was equivalent to that induced by pair feeding over the initial 6 days of exposure to either of these manipulations (Fig. 3). During the subsequent 6 days of pair feeding a plateau in weight loss was observed, while obese mice receiving ob protein continued to lose weight (Fig. 3). The overall decrease in body weight in *ob/ob* mice in response to ob protein treatment was significantly greater than that resulting from pair feeding (Table 2). Absolute fat-pad weights were significantly decreased by ob protein treatment and by pair feeding of *ob/ob* mice (Table 2). When fat pad weights were normalized to body weight, only ob protein treatment and

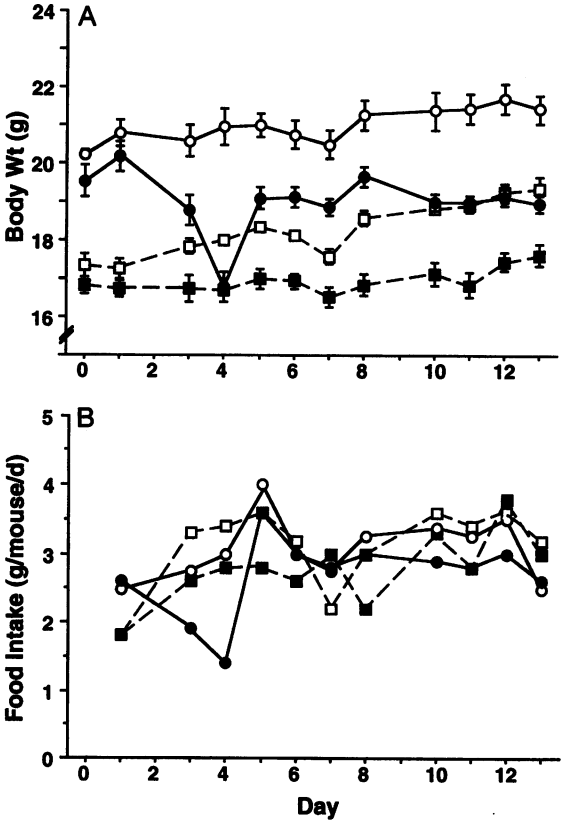

FIG. 2. Body weights (*A*) and food intake (*B*) of female lean mice (circles connected with solid lines, C57BL/6J-+/*ob*; squares connected with dashed lines, C57BL/KsJ-+/*m*) treated with ob protein (100 μg/kg per day, solid symbols) or PBS (open symbols) as described in Fig. 1. Body-weight data are the mean ± SEM. $n = 5$ per group except C57BL/KsJ-+/*m* treated with PBS, where $n = 4$. Food-intake data are the number of grams consumed per cage per day divided by the number of mice in the cage.

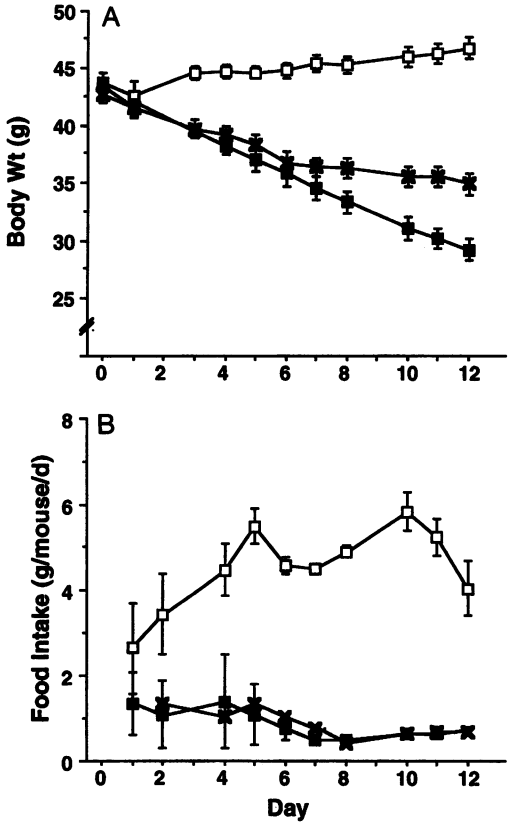

FIG. 3. Body weights (*A*) and food intake (*B*) of female obese C57BL/6J-*ob/ob* mice, treated with ob protein (270 μg/kg per day, solid squares) or PBS (open squares) as described in Fig. 1. An additional group of female obese C57BL/6J-*ob/ob* mice (x's) were treated with PBS via miniosmotic pumps and were pair-fed to the spontaneous intake of the ob protein-treated group. All data are the mean ± SEM. $n = 4$ or 5 per group for body-weight data and $n = 2$ per group for food-intake data.

Table 2. Effects of ob protein infusion (270 μg/kg per day s.c. for 14 days) or pair feeding on change in body weight, organ weights, and fasting serum end points in 8-week-old genetically obese (C57BL/6J-*ob*/*ob*) or lean (C57BL/6) mice

	Genotype					
	ob/*ob*			C57BL/6		
Treatment	PBS	ob	Pair fed	PBS	ob	Pair fed
Change in body wt*	2.3 ± 0.7	−14.2 ± 1.2‡	−6.5 ± 0.2‡§	2.5 ± 0.2	0.4 ± 0.2‡	1.0 ± 0.1‡
Absolute RP wt†	836 ± 33	386 ± 30‡	550 ± 27‡§	36 ± 4	13 ± 3‡	48 ± 5§
Relative RP wt	18 ± 1	13 ± 0.7‡	16 ± 0.6§	1.7 ± 0.2	0.6 ± 0.1‡	2.3 ± 0.3‡§
Absolute ING wt	4989 ± 175	2302 ± 182‡	3425 ± 224‡§	250 ± 11	124 ± 13‡	316 ± 20‡§
Relative ING wt	106 ± 3	78 ± 4‡	98 ± 4§	11.6 ± 0.5	6.3 ± 0.6‡	15.4 ± 0.9‡§
Absolute liver wt	3096 ± 57	1109 ± 79‡	1279 ± 26‡	1063 ± 53	960 ± 39	1015 ± 34
Relative liver wt	66.1 ± 0.9	38.1 ± 3.3‡	36.5 ± 0.3‡	49.2 ± 2.3	48.9 ± 1.9	49.7 ± 1.4
Glucose, mg/dl	702 ± 39	124 ± 11‡	175 ± 27‡	233 ± 6	160 ± 11‡	185 ± 9‡
Insulin, ng/ml	30.6 ± 6.2	0.9 ± 0.08‡	14.2 ± 4.2‡§	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.03
Cholesterol, mg/dl	275 ± 15	119 ± 12‡	153 ± 9‡	112 ± 5	88 ± 3‡	83 ± 3‡
Triglycerides, mg/dl	167 ± 22	120 ± 6	141 ± 1	105 ± 5	77 ± 5‡	94 ± 4§

All data are the mean ± SEM. n = 4 or 5 per group for *ob*/*ob* and n = 9 or 10 per group for C57BL/6.
*Grams gained or lost from day 0–14.
†Absolute organ weights expressed as mg; relative organ weights expressed as mg/g of body weight.
‡$P < 0.05$ versus PBS-treated control within genotype.
§$P < 0.05$ versus ob protein-treated within genotype.

not pair feeding resulted in significant decreases in relative fat pad weights in obese mice (Table 2).

Absolute and relative liver weights were decreased to a similar extent by ob protein treatment and by pair feeding in *ob*/*ob* mice (Table 2). Hepatic glycogen content was significantly depleted by ob protein treatment but not by pair feeding in *ob*/*ob* mice, whereas a minimal-to-moderate decrease in hepatic lipid content was observed in both ob protein-infused and in pair-fed PBS-treated *ob*/*ob* mice (data not shown). Statistically similar reductions in fasting serum glucose and cholesterol concentrations were induced by ob protein treatment and by pair feeding in *ob*/*ob* mice (Table 2). Ob protein infusions reduced fasting serum insulin levels to those observed in lean genetic controls, whereas pair feeding reduced fasting serum insulin levels by ≈50% compared to PBS-treated ad libitum-fed controls (Table 2).

There was not a statistically significant difference in the overall decrease in body weight in lean mice in response to ob protein treatment versus pair feeding (Fig. 4 and Table 2). In lean mice, absolute and relative fat-pad weights were significantly decreased by ob protein treatment but not by pair feeding, whereas relative fat-pad weights were increased in pair-fed mice relative to ad libitum-fed controls (Table 2). Neither ob protein treatment nor pair feeding affected absolute or relative liver weights in lean mice (Table 2). Hepatic lipid and glycogen contents in lean mice were also not affected by these manipulations in lean mice (data not shown). Ob protein treatment and pair feeding similarly reduced fasting serum glucose, cholesterol, and triglyceride levels in lean mice relative to ad libitum-fed PBS-treated controls (Table 2).

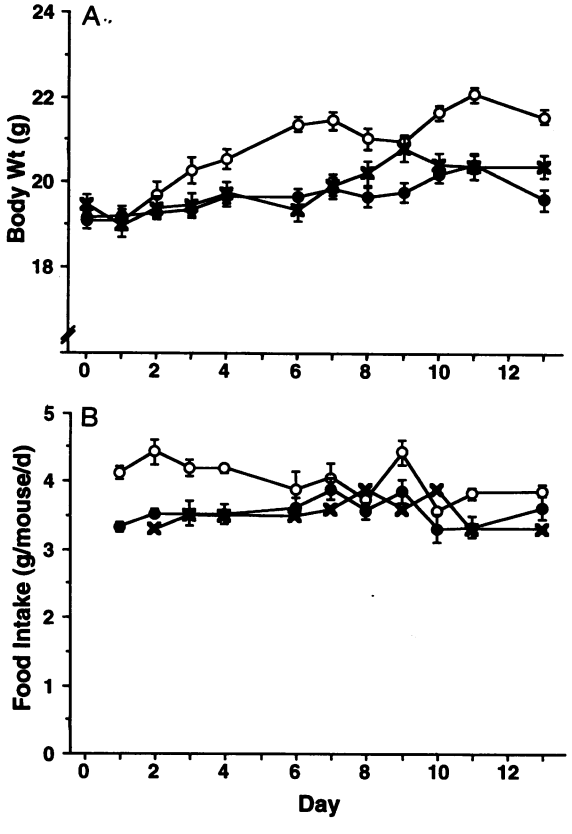

FIG. 4. Body weights (*A*) and food intake (*B*) of female C57BL/6 mice, treated with ob protein (270 μg/kg per day, solid circles) or PBS (open circles) as described in Fig. 1. An additional group of female mice (x's) were treated with PBS via miniosmotic pumps and were pair-fed to the spontaneous intake of the *ob*-treated group. All data are the mean ± SEM. n = 9 or 10 per group for body-weight data and n = 3 per group for food-intake data.

DISCUSSION

The present studies show that the significant weight- and adipose-reducing effects of the ob protein in both ob protein-deficient (*ob*/*ob*) mice and non-ob protein-deficient mice exceed those induced by food restriction paradigms that match the caloric intake of control-treated mice to that observed with ob protein treatment. These findings systematically and comprehensively compare body fat and organ weight, as well as body-weight changes in response to ob protein infusions versus paired feeding in both lean and obese mice, extending a preliminary report that pair feeding of control-treated *ob*/*ob* mice decreased body weight less than did ob protein treatment (8). The mechanism whereby the ob protein significantly reduces body-fat mass in the presence of only modest decreases in food intake in lean mice remains to be elucidated. The importance of understanding the molecular mechanism of these effects of the ob gene product is underscored by recent evidence that suggests that ob gene expression is not attenuated but rather increased in human obesity (11), implying that human obesity may be better characterized as an ob-insensitive, rather than an ob-deficient state.

While tempting to speculate that the ob protein affects fuel storage and/or energy expenditure in addition to or independent of appetite suppression, published data report that the ob protein increases oxygen consumption and body temperature in obese but not in lean mice (7). Our data and those of Halaas *et al.* (8) report similar decreases in body weight and fat in lean mice in response to ob protein, despite using different approaches to assess body fat, as well as differences in doses and routes of administration of ob protein (infused continuously at 0.1 to 0.27 mg/kg per day, s.c. in the present studies, injected twice daily at 12.5 mg/kg, i.p. in ref. 8). These findings are in contrast to the report of Pelleymounter *et al.* (7) in which decreases in carcass fat in the same strains of lean mice treated with ob protein either did not occur or were <5% (ob protein injected daily at 10 mg/kg, i.p.). In light of these as-yet-unresolved discrepancies between reports and particularly in view of the pair-feeding data in lean mice in the present studies, it seems likely that the ob protein does exert effects on metabolism in lean mice that remain to be defined experimentally.

Using lower doses and a different dosing paradigm than those previously reported, our data confirm the observation that the ob protein exerts a larger effect on food intake in the ob protein-deficient mouse than in normal lean mice. When delivered intracerebroventricularly, the ob protein inhibits food intake at a single dose of one-fourth to one-tenth of the doses delivered s.c. in the present studies (9, 12). The apparently enhanced sensitivity of the *ob/ob* mouse to ob protein, with respect to food intake, may be due to an increase in the number or signaling efficiency of receptors to the ob protein, possibly in the brain, or may reflect a difference in the pharmacokinetic properties of the ob protein between obese and lean mice.

While the existence of peripheral receptors for the ob protein cannot be ruled out at this time, the recent report that an increased expression of the ob gene in adipose tissue of mice with hypothalamic lesions does not result in a lean phenotype suggests that the ob protein does not act directly on fat cells (13). The present detailed comparison of the effects of ob protein versus pair feeding suggests that the distribution of the ob receptor is relatively restricted, as there were few profound effects of ob protein treatment on the absolute weights of numerous major organs in either lean or obese mice, except for liver. While liver weight in obese mice was similarly decreased by ≈50% in response to either ob treatment or pair feeding, histologic evidence suggests hepatic glycogen content was differentially affected by the two treatment paradigms. Quantitative analyses of these end points will help to define the precise nature and specificity of any effect that the ob protein may exert on the liver.

db/db mice were insensitive to ob protein infusions with regard to body and adipose weight, as was predicted from parabiosis studies (5, 6) and also observed in recently published reports (8, 9). The nature of the insensitivity of the *db/db* mouse to ob protein may be due to the absence of the ob receptor or a postreceptor signaling molecule (15). Elucidation of the structure and localization of the ob protein receptor and of the biological effects of the ob protein in the non-ob protein-deficient state are critical steps in determining the potential utility of the ob protein as a pharmacotherapy for the management of obesity (14).

We are grateful to M. Aguet, C. Brunet, N. Desjardin, N. Dybdal, I. Figari, G. Hatami, M. Jones, Y. Ma, J. Mather, S. Shak, S. A. Spencer, and D. Yansura for their invaluable assistance and critical input to the project.

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