Synergy of Sibutramine and Low-Dose Leptin in Treatment of Diet-Induced Obesity in Rats

Carol N. Boozer, Rudolph L. Leibel, Robert J. Love, Ming C. Cha, and Louis J. Aronne

Tachyphylaxis to the effects of anorexigenic agents, such as sibutramine (S), may be due, in part, to counterregulatory decreases in energy expenditure (EE) and increases in hunger that result from reduced circulating leptin (L) due to loss of body fat and lowered L production/adipocyte. The present study was conducted to test the hypothesis that L administered at low doses sufficient to restore ambient L to preweight loss concentrations would enhance the intercurrent efficacy of S by reducing the strength of physiologic counterregulation to weight loss. Forty male Sprague-Dawley rats were fed a high-fat (HF) diet (45% energy) to induce obesity. After 8 weeks, the obese rats (600 ± 58 g) were weight-matched into 4 groups (N = 8/group) and implanted subcutaneously (SC) with 2 mL, 7-day Alzet mini-pumps that provided: vehicle (V, saline), L (0.5 mg/kg/d), S (3 mg/kg/d), or L+S. Food intake (FI) on the HF diet was measured daily. On day 7, 24-hour EE was measured by indirect calorimetry, and the animals then killed for body composition analysis. Compared with vehicle, treatment with S alone, but not L alone, produced significant weight loss (-23 ± 26 *v* -6 ± 16 g, *P* < .01). L alone, or with S, increased fat oxidation (decreased respiratory quotient [RQ]) compared with V (*P* < .05). The lack of decline in EE with S may be due to its documented effect to stimulate thermogenesis. Administration of L with S synergistically decreased FI and increased weight loss and fractional fat loss. A reduction in plasma L concentration may contribute to the "plateau phenomenon" observed in studies of weight loss therapies. Replacement doses of L during S administration increased weight loss and fractional fat loss by (1) decreasing food intake and (2) by increasing fat oxidation. Such drug combinations may be useful in the treatment of human obesity.

LEPTIN (L) CLEARLY PLAYS a role in energy homeostasis as demonstrated by effects of L axis mutations in rodents and humans.[1] One model for L's function is that it serves primarily to signal sufficiency of fat stores for metabolic homeostasis and reproduction. Relative deficiency leads to increased food intake, decreased energy expenditure (EE), infertility, and decreased growth.[2,3]

Blood levels of L are closely proportional to body fat,[4,5] but decline acutely with decreased caloric intake.[2,6-8] In rats, L administered intracerebrally, prevented the decrease in EE that occurred in pair-fed, sham-infused animals,[9] but it is unclear whether circulating concentrations of L account for variations in EE in humans.[8,10,11]

Premenopausal females have circulating concentrations of L/fat mass that are approximately 3-fold higher than in males, yet are not anorectic.[5] Very high doses of exogenous L are required to induce weight loss in humans.[12] Even then, the effects on body weight composition are unpredictable and vary greatly among individuals. L appears to monitor insufficiency of fat stores more closely than excess and may function primarily to defend body fat stores.[2,3,13] Individual thresholds to L action (hence different minima for body fat) may depend on genes relating to L transport and signal transduction in the hypothalamus.[14] At usual body weight, obese and lean are in energy equilibrium at different circulating concentrations of L.[4,5] Weight loss leads to decreased body fat resulting in decreased L concentrations in blood and brain, which signal that fat stores are diminished.[15]

Based on these considerations, L may be more useful therapeutically as a replacement hormone, than as an inducer of weight loss. The present experiment was designed to test this possibility by combining low-dose "replacement," levels of L combined with an anorectic agent, sibutramine (S).

MATERIALS AND METHODS

Animals and Diets

Five groups of rats were studied: 1 lean group fed laboratory chow, and 4 obese groups fed a high-fat (HF) diet. The obese rat treatment groups were vehicle, S, L, and the combination of S plus L. Fifty male, Sprague-Dawley rats (400 ± 10 g) were purchased from (Charles River Laboratories, Raleigh, NC) and individually housed throughout the study in hanging stainless steel cages in a room with temperature (23 ± 2°C), humidity (50%), and light control (12:12-hour light:dark). Water and food were provided ad libitum throughout the study. After 1 week of habituation to a commercial rat chow (Lab Diet, PMI Nutrition International, St Louis, MO), 40 of the rats were switched to a diet enriched with fat to promote obesity. The remaining rats (lean, N = 10) were fed laboratory chow throughout the study to provide a reference for body composition of the rats in the 4 treatment groups. The higher fat diet (45% fat, 20% protein, and 35% carbohydrate by energy) was prepared by mixing 282 g Crisco vegetable shortening and 55 g casein (Teklad Premier Laboratory Diets, Madison, WI) per kilogram of ground rat chow in a commercial mixer (Hobart, Long Island City, NY). Eight weeks after initiation of the HF diet, the obese rats (obese) (600 ± 58 g) were weight-matched into 4 groups (8 rats/group) and given subcutaneous infusion for 7 days of either vehicle (saline), murine L (PeproTech, Rocky Hill, NJ), S (Knoll Pharmaceutical, Cedar Knolls, NJ), or both L and S by minipumps. Tail blood was collected from all animals before pump implantation. Body weight and food intake (corrected for spillage) were measured daily. Six rats/obese experimental group had 24-hour EE measured by indirect calorimetry

From the New York Obesity Research Center (NY ORC), St. Luke's-Roosevelt Hospital Center, New York; Institute of Human Nutrition and Department of Medicine, and Department of Pediatrics and Naomi Berrie Diabetes Center, Columbia University; New York; and the Weill-Cornell Medical College, New York, NY.

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Address reprint requests to Carol N. Boozer, DSc, WH1029, St. Luke's-Roosevelt Hospital Center, 1111 Amsterdam Ave, New York, NY 10025.

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before implantation of minipumps, and again from days 6 to 7 after implantation. On day 7 after implantation, all rats, including lean rats, were lightly anesthetized with carbon dioxide gas and blood collected by cardiac puncture before euthanasia by overdose of carbon dioxide. Adipose depots (inguinal, mesenteric, epididymal, and retroperitoneal) were excised and weighed, then returned to carcasses. Gastrointestinal tracts were removed, cleaned, and returned to carcasses, which were frozen (-10°C) until body composition determination by direct chemical analysis.

The study was approved by the Institutional Animal Care and Use Committee, and all animals were cared for according to approved institutional guidelines.

Subcutaneous Infusion

Alzet minipumps (Alza, Palo Alto, CA) Model 2002 and 2ML1 were used as the delivery system. L was dissolved in sodium citrate solution (10 mmol/L, pH 4.0) at a concentration designed to deliver 0.5mg/kg/d (Model 2ML1 minipumps with 2.1 mL capacity, 235 μL/d rate). S was dissolved in saline at a concentration to deliver 3 mg/kg/d (Model 2002 minipump, 242 μL capacity, 13.2 μL/d rate). Animals in the dual L/S treatment group were implanted with 2 pumps to avoid mixing of the 2 agents in the delivery device. Pumps were soaked in saline (0.9% NaCl, 37°C) for 1 hour before implantation. After light anesthesia with halothane (Fluothane; Ayerst Laboratories, Philadelphia, PA), the interscapular region of the rat was shaved, a 1-cm incision was made, tissues were spread gently by hemostat, and the pump inserted in the resulting subcutaneous space. Skin was sutured with wound clips.

EE

Twenty-four hour EE was assessed by indirect calorimetry, as previously described.[9] The calorimeter system consists of a Magnos IV oxygen analyzer and a Uras 3G carbon dioxide analyzer (both by Hartmann Braun, Frankfurt, Germany), and 6 Plexiglas metabolic chambers maintained in an environmental room that is separate from the analyzers. First pass fresh air, maintained at constant temperature and humidity, is continuously pulled through the chambers by individual vacuum pumps located outside the environmental room. Airflow is set at 1.5 to 1.8 L/min by individual flow controllers (Columbus Instruments, Columbus, OH) placed between the pumps and the chambers. The exact flow rate at each controller is electronically recorded to computer simultaneously with sampling of the corresponding chamber. Chambers are serially sampled by the system computer program. Sampled air is then pulled through a condenser (Hartmann Braun) to remove moisture before analysis. Percentage of oxygen consumed and carbon dioxide produced is converted to milliliters/minute by multiplying fractional content of the respective gas by the flow rate corrected to standard temperature and pressure. EE is calculated by standard formulae[16] from 23 hours of collected data, then extrapolated to 24 hours. The analyzers are calibrated by diluting room air with CO_2 or nitrogen using a Westoff gas mixing pump (Digamix, Type m/300c, Bochum, Germany). The entire system, tested by burning propane, has an estimation error of less than 1%.

Plasma L Analysis

L concentration of plasma was assayed in duplicate by the Hormone/Metabolite Core Laboratory of the New York Obesity Research Center using radioimmunoassay kits (Linco Research, St Charles, MO). For the L assay, sensitivity is 0.5 ng/mL, the limit of linearity is 50 ng/mL (100-μL sample size) and the coefficient of variation (CV) is 8.8%.

Body Composition Analysis

Carcass composition analysis was performed in the Animal Body Composition Core Laboratory of the New York Obesity Research Center. For analysis, frozen carcasses were autoclaved (125°C) in 500 mL distilled water for 1 hour in large beakers with covered tops, then homogenized with a large-bore Polytron (PT 6000; Brinkman Instrument, Westbury, NY). After weighing, aliquots (in duplicate) were dried to stable weight to determine water content. Total carcass lipid was determined by chloroform:methanol extraction of homogenate samples.[17] Nitrogen content was determined by an adaptation[18] of the Kjeldahl method[19] and protein calculated, assuming a N/protein ratio of 0.16. From these measurements, water content, fat mass, and fat-free mass were calculated.

Statistics

Data are presented as mean ± SD in tables and mean ± SEM in figures. The effect of the treatments was determined by 1-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (LSD) test to determine differences between means using the SPSS general linear model program (version 7.0, SPSS, Chicago, IL). Differences between means were considered to be significant at $P < .05$.

RESULTS

Body Weight

Body weight changes of the 4 obese groups of rats over 6 days of treatment are shown in Fig 1. Body weight change was not significant in either the vehicle or the L group and did not differ between these 2 groups. Body weight loss of the S group was significantly greater ($P < .05$) than both vehicle and L groups after 2 days of treatment. The S plus L combination group (Sib/Lep) lost significantly more ($P < .05$) weight than vehicle or L groups after only 1 day of treatment. Compared with the vehicle group (which gained 5 ± 9 g), the change in body weight over the 6 days of treatment was significantly increased ($P < .01$) by S (-23 ± 26 g) alone, but not by L alone (-6 ± 16 g). Change in body weight in the Sib/Lep combination

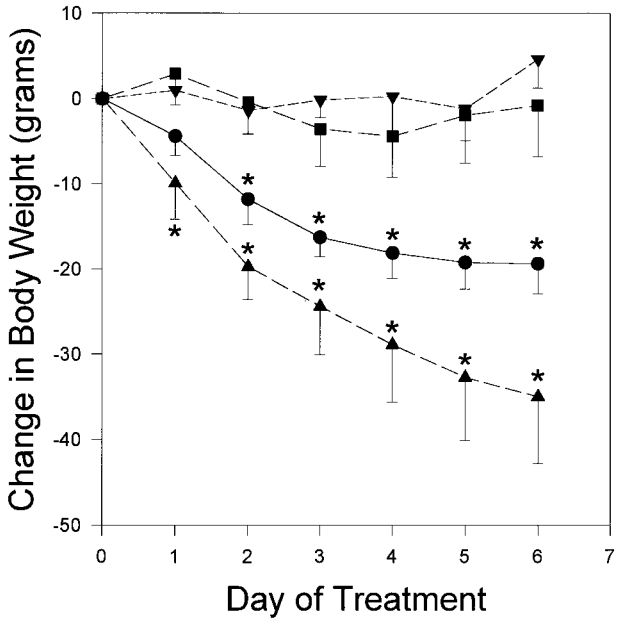

Fig 1. The changes in body weight of rats during 6 days of treatment with vehicle (▼, N = 7), L (■, N = 8), S (●, N = 8) or S plus L (▲, N = 8). * Significantly different ($P < .05$) from vehicle and L.

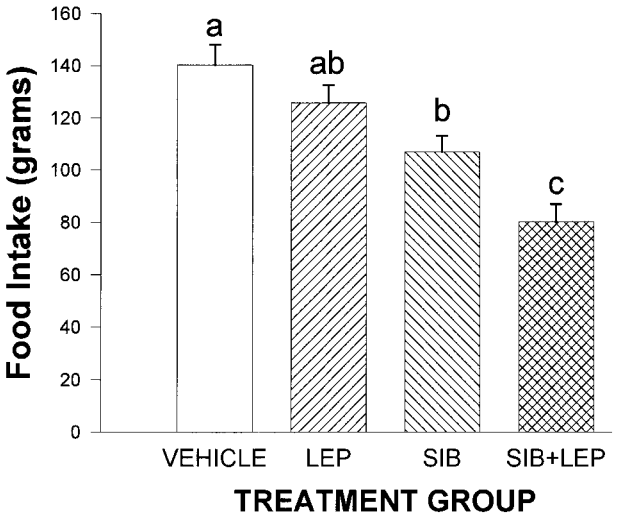

Fig 2. Total food intake (in grams) over 7 days of treatment with vehicle, N = 7, L (Lep, N = 8), S (Sib, N = 8), or combination treatment with S plus L (Sib/Lep, N = 8). Different letters indicate that means are significantly ($P < .05$) different.

group (-35 ± 18 g) was significantly greater ($P < .01$) than that of either the vehicle or L group and was nearly significantly greater ($P < .056$) than S alone.

Food Intake

Compared with the vehicle group (140 ± 20 g), total food intake during the treatment period was unaffected by the L treatment (126 ± 19), but was significantly lower ($P < .05$) in the S group (107 ± 17 g) (Fig 2). Food intake of the Sib/Lep group (80 ± 19 g) was significantly lower than any other group. The combination of S plus L synergistically reduced mean food intake since the reduction in food intake in this group compared with vehicle (-60 g) was greater than the sum of reductions in the groups tested with L (-14 g) and S alone (-33 g).

EE and Respiratory Quotient

Twenty-four hour EE was not significantly different among groups either before or after treatment (Table 1). All groups had a tendency to lower EE after treatment, but there were no significant differences among groups in the amount of change. EE/100 g of lean body mass also did not differ among groups. Mean 24-hour respiratory quotient (RQ) did not differ among the groups before treatment, but at day 7 of treatment, RQ was significantly lower ($P < .05$), indicating greater oxidation of fat in both the L group (0.77 ± 0.03) and the Sib/Lep group (0.76 ± 0.02) compared with the vehicle group (0.81 ± 0.02). The change in RQ with treatment, however, was significantly greater ($P < .05$) than vehicle (0.00 ± .07) only in the Sib/Lep combination group (-0.07 ± .03).

Body Composition

Total carcass fat relative to vehicle group mean (104 ± 45 g) was numerically reduced with L (81 ± 28 g) and S (86 ± 16 g), but only the combination Sib/Lep (66 ± 16 g) was statistically significantly different ($P < .01$) from vehicle (Table 2). The lean, never obese, group also had significantly less carcass fat than the HF-fed vehicle group (62 ± 15 *v* 104 ± 45 g, $P < .005$). Carcass composition of the Sib/Lep group was very similar to that of the lean, never-obese, group in terms of body fat, as well as protein and water, whether expressed absolutely, or as a percent of total carcass weight. Protein and water expressed absolutely did not differ among the groups, so that, in general, any significant differences between groups in fractional protein and water content reflected the differences in absolute amounts of body fat.

Each adipose depot weighed, with the exception of epididymal, was significantly smaller ($P < .05$) in the combination Sib/Lep group, when compared with the vehicle group (Fig 3). Individual adipose depot weights of the lean group were all significantly less ($P < .05$) than equivalent weights in the vehicle group, but were similar to weights in the Sib/Lep group. No other comparisons between groups were significantly different.

Plasma L

Plasma L concentrations were not significantly different among obese rat groups, either before or after treatment (Table 3). Expressed relative to total body fat mass, these concentrations also did not differ between groups (3.7 ± 0.2, 3.7 ± 0.1, 2.8 ± 0.1, and 3.3 ± 0.2 μg/dL/100 g carcass fat for vehicle, L, S, and Sib/Lep treatment groups, respectively). The absence of significantly higher plasma L values in the 2 L-treated groups may be due to reduced carcass fat secondary to L administration and/or to suppression of endogenous L production by L-mediated activation of the sympathetic nervous system[20,21] or to direct effects of administered L on adipocyte expression or release of L.[22] S administration would also be expected to reduce plasma L concentrations by mechanisms related to its effects on the sympathetic nervous system.[23]

DISCUSSION

The treatment of obesity by conventional combinations of diet, exercise, and anorexiants can produce modest degrees of

Table 1. EE (kcal/d) and RQ Before and After Seven Days of Treatment

	EE-0	EE-7	ΔEE	EE-7/LBM	RQ-0	RQ-7	ΔRQ
Vehicle	98 ± 6	91 ± 16	−6 ± 11	22 ± 2	0.81 ± .07	0.81 ± .02[a]	0.00 ± .07[a]
L	98 ± 11	93 ± 5	−5 ± 12	22 ± 2	0.81 ± .05	0.77 ± .02[b]	−0.04 ± .04[ab]
S	98 ± 10	91 ± 6	−6 ± 9	22 ± 1	0.84 ± .03	0.79 ± .02[ab]	−0.05 ± .05[ab]
S + L	103 ± 10	89 ± 6	−14 ± 7	21 ± 0	0.84 ± .04	0.76 ± .02[b]	−0.07 ± .03[b]

NOTE. EE-0, EE before treatment; EE-7, EE on day 7 of treatment; ΔEE, change in EE, day 0 to day 7; EE-7/LBM, EE on day 7 of treatment/100 g of lean body mass. Different letters indicate that means are significantly different ($P < .05$).

Table 2. Carcass Composition of Rats After Seven Days of Treatment With Either Vehicle, L, S, or S + L

	Weight (g)	Fat (g)	Protein (g)	Water (g)
Lean	497 ± 24[a]	62 ± 15[a]	112 ± 8	313 ± 24
		12 ± 3%[a]	23 ± 1%[a]	63 ± 3%[a]
Vehicle	536 ± 88[b]	104 ± 45[b]	106 ± 16	303 ± 34
		19 ± 7%[b]	20 ± 1%[b]	57 ± 6%[b]
L	531 ± 54[ab]	81 ± 25[ab]	113 ± 12	322 ± 33
		15 ± 4%[ab]	21 ± 1%[c]	61 ± 4%[ab]
S	527 ± 44[ab]	86 ± 15[ab]	117 ± 22	313 ± 24
		16 ± 2%[ab]	21 ± 1%[bc]	59 ± 1%[b]
S + L	508 ± 36[a]	66 ± 16[a]	114 ± 11	315 ± 24
		13 ± 3%[a]	22 ± 1%[ac]	62 ± 3%[ab]

NOTE. Different letters indicate that means are significantly different ($P < .05$).

weight reduction that are difficult to maintain over extended periods of time.[24] One of the reasons for the high recidivism encountered in most such treatment paradigms is that the weight loss (reduction in body fat) results in compensatory alterations in appetite and EE that tend to restore body fat to its prereduction status.[25]

The physiology and molecular genetics of L renders this molecule an important candidate for mediating some aspects of systemic alterations of energy homeostasis in the context of experimentally or therapeutically reduced body weight.[3] Hormonal and neuroendocrine responses to fasting, such as suppression of T_4, decreased hypothalamic expression of proopiomelanocortin (POMC) and cocaine- and amphetamine-related transcript (CART) mRNAs, and increased hypothalamic expression of neuropeptide Y (NPY) mRNA, are all prevented by infusion of L during fasting.[2]

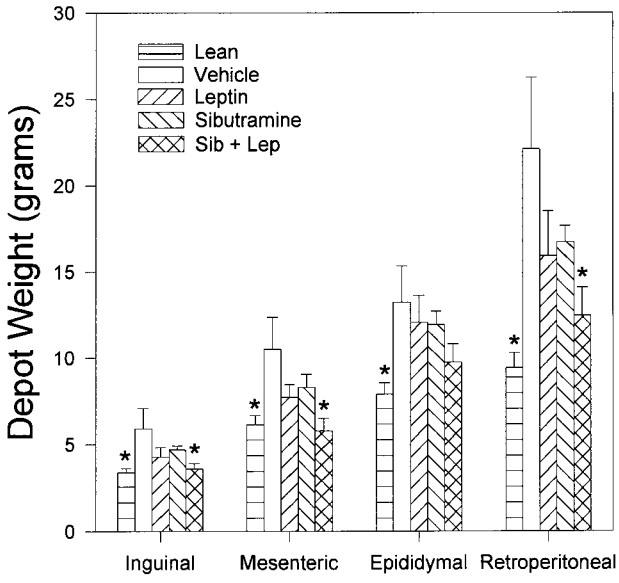

Fig 3. Adipose depot weights (in grams) of lean (never obese) rats (N = 8) and obese rats treated with vehicle (N = 7), L (Lep, N = 8), S (Sib, N = 8), or combination treatment with S plus L (Sib/Lep, N = 8). * Indicates that means are significantly ($P < .05$) different from vehicle-treated rats.

Table 3. Plasma L Concentrations (ng/mL) of Obese Rats Before and After Seven Days of Treatment With Either Vehicle, L, S, or S + L

	Day 0	Day 7	Change
Vehicle	2.07 ± 0.63 (4)	2.65 ± 1.40 (8)	1.77 ± 0.51 (4)
L	1.68 ± 0.96 (4)	2.97 ± 1.17 (8)	1.47 ± 1.18 (4)
S	2.01 ± 0.36 (8)	2.38 ± 0.24 (8)	0.37 ± 1.44 (8)
S + L	1.96 ± 1.31 (8)	2.09 ± 0.92 (8)	0.12 ± 1.80 (8)

NOTE. Number/group indicated in parentheses (numbers are not additive for vehicle and L groups due to incomplete data at day 0). There were no statistically significant effects of time or treatment.

The experiment reported here tests the possibility that intercurrent provision of low doses of L sufficient to restore circulating concentrations to those present before the initiation of weight reduction will mitigate some of the otherwise vigorous metabolic and behavioral resistance to an extended state of negative energy balance.[25] The combination of low-dose ("replacement") L with an anorexiant dose of S, administered to animals provided with a palatable, HF diet, was intended to mimic a clinical circumstance in which low-dose L might have utility.

The dose of L was sufficient, by itself, to cause decreases in food intake (-14%), carcass fat (-22%), and RQ (-5%), in comparison to vehicle-treated animals. S alone produced changes of -24%, -17%, and –2%, respectively, in these parameters. When used at these doses in combination, the 2 molecules had clear synergistic effects on food intake, body weight and fractional fat loss, as well as fat oxidation and loss of fat from specific depots (inguinal, mesenteric, and retroperitoneal). These effects may have been mediated by L's effects to initiate the compensatory hormonal and neuroendocrine changes that are secondary to the anorexic and weight loss effects of S.[2] L's enhancement of S's effects to reduce food intake, while maintaining EE, produced greater energy balance deficits demonstrated here by increased fat oxidation (decreased RQ), decreased body fat, and body weight in the Sib/Lep group.

We predicted that L would prevent the decline in EE that accompanies weight loss, and this prediction was confirmed. The fact that animals treated with S alone showed a similar maintenance of 24-hour EE is consistent with previous reports that this compound enhances systemic sympathetic nervous activity.[24]

The present study found that replacement doses of L during S administration synergistically decreased food intake and increased weight loss and fractional fat loss. These results, in agreement with previous observations,[9,26] suggest that the decline in circulating L that accompanies therapeutic reduction in body fat mediates some of the behavioral and metabolic counterregulation to that process. These results also suggest that some of the well-recognized apparent tachyphylaxis to catechol/serotonin-related anorexiants, such as S,[27] may be secondary to changes in hypothalamic neuropeptides in response to reduced concentrations of L. We postulate that a reduction in serum L concentration may be the cause of the "plateau" phenomenon observed in studies of weight loss therapies in both humans and animals.[27-30] The synergy between an oral anorexiant of modest potency, and low-dose (replacement) L suggests a therapeutic strategy for long-term clinical manage-

ment of obesity. For example, such combination therapy might be used to induce weight reduction to the desired level, and L alone continued in low dose to sustain the weight reduction indefinitely. This strategy is readily testable in humans.

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