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Amylin-Mediated Restoration of Leptin Responsiveness in Diet-Induced Obesity: Magnitude and Mechanisms

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Previously, we reported that combination treatment with rat amylin (100 μg/kg·d) and murine leptin (500 μg/kg·d) elicited greater inhibition of food intake and greater body weight loss in diet-induced obese rats than predicted by the sum of the monotherapy conditions, a finding consistent with amylin-induced restoration of leptin responsiveness. In the present study, a 3 × 4 factorial design was used to formally test for a synergistic interaction, using lower dose ranges of amylin (0, 10, and 50 μg/kg·d) and leptin (0, 5, 25, and 125 μg/kg·d), on food intake and body weight after 4 wk continuous infusion. Response surface methodology analysis revealed significant synergistic anorexigenic ($P < 0.05$) and body weight-lowering ($P < 0.05$) effects of amylin/leptin combination treatment, with up to 15% weight loss at doses considerably lower than previously reported. Pair-feeding (PF) experiments demonstrated that reduction of food intake was the predominant mechanism for amylin/leptin-mediated weight loss. However, fat loss was 2-fold greater in amylin/leptin-treated rats than PF controls. Furthermore, amylin/leptin-mediated weight loss was not accompanied by the counterregulatory decrease in energy expenditure and chronic shift toward carbohydrate (rather than fat) utilization observed with PF. Hepatic gene expression analyses revealed that 28 d treatment with amylin/leptin (but not PF) was associated with reduced expression of genes involved in hepatic lipogenesis (Scd1 and Fasn mRNA) and increased expression of genes involved in lipid utilization (Pck1 mRNA). We conclude that amylin/leptin interact synergistically to reduce body weight and adiposity in diet-induced obese rodents through a number of anorexigenic and metabolic effects. (*Endocrinology* 149: 5679–5687, 2008)

THE CENTRAL NERVOUS system receives, integrates, and responds to many hormonal signals from the periphery and is recognized as the key regulator of long-term energy homeostasis. Advances in our understanding of the integrated neurohormonal regulation of food intake and body weight have provided a sound scientific basis for novel approaches to obesity drug development (1, 2). For instance, there is growing recognition that the modest weight loss elicited by any single agent is likely limited not only by the redundancy of regulatory feeding pathways but also by the fact that initial weight loss activates powerful compensatory mechanisms aimed at defending initial body weight (weight loss counterregulation) (1–3). Thus, combination approaches aimed at harnessing the interaction of islet-, adipose-, and/or gut-derived signals may be one way to overcome these compensatory mechanisms (1, 2).

Amylin is a peptide hormone that is cosecreted with insulin from pancreatic β-cells in response to nutrient ingestion. Amylin binds amylin receptors in the hindbrain area postrema, which resides outside the blood-brain barrier, activating multiple central nervous system regions implicated in glucose and energy homeostasis (4, 5). In obese humans, administration of the amylin analog pramlintide reduced caloric intake and elicited sustained weight loss (6–9). In diet-induced obese (DIO) rats, amylin-induced weight loss was primarily attributable to reduced food intake and was fat specific (10–12).

Leptin is a cytokine hormone produced in adipose tissue that plays a critical role in regulating energy homeostasis. Although increased fat mass is associated with increased circulating leptin concentrations, obesity is also associated with leptin resistance. Thus, although leptin replacement elicits profound weight-reducing effects in leptin-deficient mice and humans, pharmacological administration of leptin to DIO rodents has demonstrated only marginal, if any, effects on body weight (13–16). Consistent with these findings in DIO preclinical models, numerous monotherapy studies with the leptin analog meterleptin in obese subjects have failed to show significant weight loss (data on file, Amylin Pharmaceuticals, Inc.).

Recent combination studies in leptin-resistant DIO rats demonstrate that peripheral administration of amylin restores leptin sensitivity (17). Specifically, infusion of an ineffective dose of leptin (500 μg/kg·d, no significant effect on body weight) combined with a moderately effective dose of amylin (100 μg/kg·d, 6% weight loss) elicited marked, synergistic weight loss (12%) (17). This same dose of leptin conferred no additional weight-reducing effects when administered to a group of DIO rats that were pair fed (PF) to the amylin-treated group, implying that amylin/leptin synergy for body weight loss may not merely be explained by the anorexigenic effects of amylin (17). The clinical significance of these findings was established in a recent translational clinical research study. In overweight/obese humans, 20 wk pramlintide/meterleptin combination treatment, after 4 wk pramlintide monotherapy, induced significantly

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Abbreviations: DIO, Diet-induced obese; PF, pair fed; RER, respiratory exchange ratio; RSM, response surface methodology.

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greater weight loss (12.7%) than treatment with pramlintide or meterleptin alone (8.4%) (17).

The present series of studies aimed to broaden our understanding of the dose response of amylin/leptin interaction as well as the mechanisms responsible for amylin/leptin-mediated weight loss in DIO rats. First, we evaluated body weight and food intake responses (over 28 d) in DIO-prone rats exposed to a lower range of dose combinations of amylin (0, 10, and 50 μg/kg·d) and leptin (0, 5, 25, and 125 μg/kg·d) than previously examined and used response surface methodology (RSM) to formally test for statistical synergy between amylin and leptin. In a second series of studies, we compared body weight, energy expenditure, plasma parameters, and hepatic gene expression in animals exposed to 4 wk treatment with amylin (50 μg/kg·d) plus leptin (125 μg/kg·d) and PF controls to assess whether amylin/leptin-mediated weight loss was associated with changes beyond those predicted by caloric restriction alone.

Materials and Methods

Experimental animals

All studies were approved by the Institutional Animal Care and Use Committee at Amylin Pharmaceuticals, Inc., in accordance with Animal Welfare Act guidelines. Animals were housed individually in standard caging at 22 C in a 12-h light, 12-h dark cycle. For RSM analysis in animals exposed to varied combinations of amylin/leptin, inbred male DIO-prone rats were obtained from Charles River Laboratories (Wilmington, MA). These rats were developed from a line of Crl:CD(SD)BR rats that are prone to become obese on a diet relatively high in fat and energy (18) and are referred to as DIO-prone rats throughout the manuscript. DIO-prone rats were maintained *ad libitum* on a moderately high-fat diet (32% kcal from fat, D1226B; Research Diets, New Brunswick, NJ) for 6 wk before and during treatment. The second series of studies comparing amylin/leptin treatment to PF controls used outbred Sprague Dawley rats (Charles River Laboratories) maintained on the same 32% fat diet (D1226B; Research Diets) and are referred to as DIO rats throughout the manuscript.

Peptides

Rat amylin (Peptisyntha, Torrance, CA) was dissolved in vehicle (50% dimethylsulfoxide in sterile water). Murine leptin (Amylin Pharmaceuticals, Inc., San Diego, CA) was resuspended in sterile water. Using aseptic technique, all rats were implanted with two ALZET osmotic mini-pumps (DURECT Corp., Cupertino, CA) containing either drug or the appropriate vehicle.

Studies and peptide administration

In the first series of studies, which investigated amylin/leptin interaction using RSM, amylin was infused at a rate of 0, 10, or 50 μg/kg·d, and leptin was infused at a rate of 0, 5, 25, or 125 μg/kg·d. Amylin and leptin were administered for 4 wk alone, and in combination, using a 3 × 4 factorial design for a total of 12 treatment groups (n = 5 per group; starting body weight = 463 ± 4 g).

Based on the marked weight loss synergy observed with 50 μg/kg·d amylin plus 125 μg/kg·d leptin in the first series of studies, this dose combination was selected for further mechanistic studies. Male DIO rats (n = 10 per group; mean starting body weight = 476 ± 4 g) were divided into one of three treatment groups: vehicle, amylin/leptin, or PF. All rats were implanted with two separate osmotic pumps. In vehicle and PF control rats, both pumps contained vehicle, whereas amylin+leptin-treated rats were implanted with one pump containing amylin (50 μg/kg·d) and one pump containing leptin (125 μg/kg·d). Although vehicle and amylin/leptin-treated rats had *ad libitum* food access, PF controls were restricted to the mean daily food intake of the amylin/leptin-treated group. Body weight and food intake were recorded daily and food was presented to the PF group between 1500 and 1600 h daily. Energy intake relative to body weight was calculated as the amount of food consumed (in kilojoules) divided by the mean body weight in grams for a 24-h period.

Body composition

For determination of fat and lean mass, rats were briefly placed (~1 min) in a ventilated Plexiglas tube that was then inserted into a rodent nuclear magnetic resonance machine (Echo Medical Systems, Houston, TX). Rats were scanned before pump implantation and on the final day of the experiment after removal of the mini-pumps. The change in adiposity (percent body fat) and dry lean tissue was calculated as previously described (12).

Plasma analysis

Terminal plasma leptin and amylin concentrations were measured by ELISA (Linco-Millipore, Billerica, MA) after 4 wk treatment in the dose-response study. For the amylin/leptin *vs.* PF study, animals were fasted overnight at d 13 for whole-blood glucose analysis using a glucometer (OneTouch Ultra; LifeScan, Milipitas, CA) on d 14. On d 28, measurements of blood glucose, plasma triglyceride, total cholesterol, and high-density lipoprotein cholesterol levels were performed (Olympus AU400e Bioanalyzer). Plasma insulin was measured from blood collected on d 14 and 28 using an ELISA (Crystal Chem, Downer's Grove, IL).

Energy expenditure and locomotor activity

Measurement of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) was performed over a period of 24 h, after 24–48 h acclimation, using an Oxymax indirect calorimeter (Columbus Instruments, Columbus, OH) on d 4 and 19 (n = 5 per group) for vehicle, amylin/leptin, and PF rats. Animals were given *ad libitum* access to food (except for PF controls, fed approximately 1 h before lights off) while in the metabolic chambers. Activity levels over 24 h (beams broken in x- and y-axes) were recorded in a second subset (n = 5 per group) on d 5 (MotorMonitor; Hamilton Kinder, Julian, CA).

Histology

The retroperitoneal fat pad was dissected, frozen on powdered dry ice, and stored at −80 C. Cryostat sections (16 μm; Leica; cooled to −30 C), were collected on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and dried at room temperature. Hematoxylin and eosin (Richard Allen Scientific, Kalamazoo, MI) staining was performed using the supplier's protocols. Slides were imaged using conventional bright-field microscopy (DM5000; Leica). Minor adjustments were applied equally across all images in Adobe Photoshop, CS2 (n = 4 per group).

Tissue gene expression

Liver and brown adipose tissue was excised at termination, snap frozen in liquid nitrogen, and stored at −80 C. Total RNA was extracted using TRI Reagent (Ambion, Austin, TX) and cDNA generated using the RETROscript RT-PCR RT system (Ambion). Quantitative real-time PCR (ABI PRISM 7900 Sequence Detection System; Applied Biosystems, Foster City, CA) for genes encoding fatty acid synthase (*Fasn*), phosphoenolpyruvate carboxykinase (*Pck1*), uncoupling protein-1 (*Ucp1*), and 18S rRNA housekeeping gene were performed using TaqMan gene expression assays-on-demand and Universal PCR master mix (Applied Biosystems). Primers and probe for stearoyl-coenzyme A desaturase-1 were sense, 5′-tcgccactgacttgctatgttatc-3′; antisense, 5′-ggaggttcttgggatgattaaagg-3′; and probe, 5′-6FAM- ctcttgtaacaaaccc-TAMRA-3′ (Applied Biosystems).

Data analysis

For RSM analysis, a first-order plus interaction model was fit to the observed percentage vehicle-corrected change in body weight. This model contains effects for the intercept, linear effect of amylin, linear effect of leptin, and linear interaction effect for amylin/leptin. A second-order model was fit to the observed percentage vehicle-corrected food intake. This model contains effects for the intercept, linear and quadratic

effects of amylin, linear effect of leptin, and linear interaction effect for amylin/leptin. The quadratic effect for leptin was unnecessary and was excluded from this model. Using these models, a response surface that predicted weight loss and food intake over the entire dose region was created.

All other parameters were compared using one-way ANOVA with contrasts. A multiple comparison adjustment was not made due to the small sample size. For gene expression, a $\log_{10}$ transformation was applied when needed to ensure homogeneity of variance. An outlying data point was excluded if its Studentized residual was more than 3 SD from the mean. Statistical analyses were performed using SAS version 8.2 (SAS Institute, Inc., Cary, NC), and the significance level was 0.05. Graphs were generated using Prism 4 for Windows (GraphPad Software, San Diego, CA). All data points are expressed as mean ± SEM.

Results

Amylin and leptin synergistically reduced body weight and food intake (RSM)

After 4 wk treatment, RSM analysis of change in body weight (vehicle-corrected) revealed a significant dose-response effect for amylin alone ($P < 0.0001$; Fig. 1A) and significant synergy between amylin and leptin ($P < 0.05$; Fig. 1A and Table 1). The dose response on body weight of leptin alone was not statistically significant. In the RSM framework, statistical synergy is indicated when the slope of the surface changes as the doses are increased; in this case, the surface became steeper (*i.e.* weight loss accelerated) as the doses of amylin and leptin were increased (Fig. 1B). The first-order plus interaction model fit to these data had a nonsignificant lack of fit, indicating that a higher-order polynomial model was unnecessary to adequately model the data. The r^2 value of the model was 0.55.

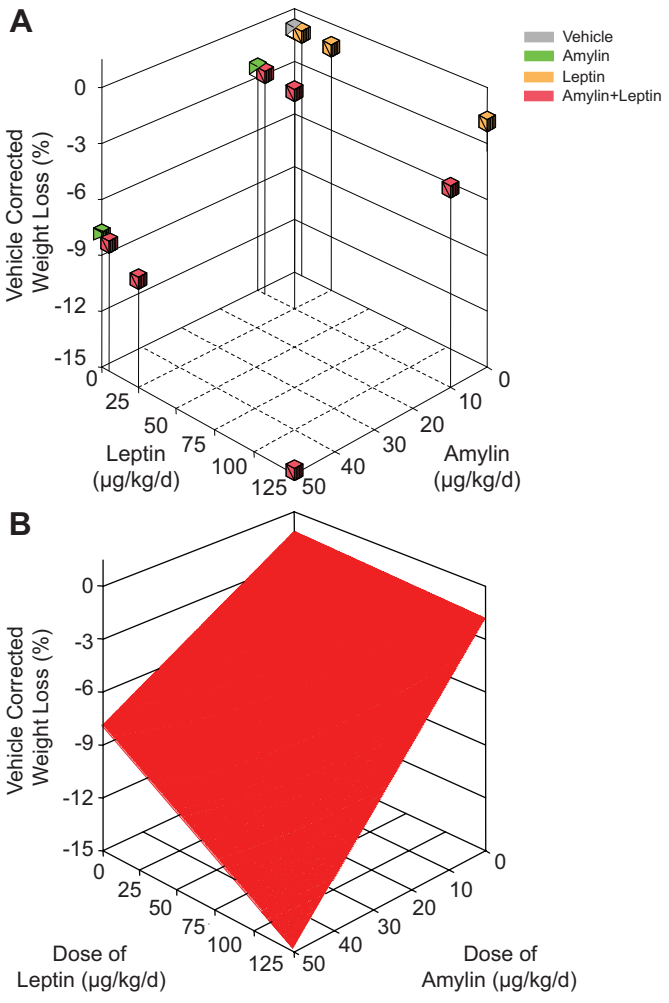

FIG. 1. A, Effects of amylin, leptin, and amylin/leptin on body weight; B, predicted response surface after 28 d exposure in DIO-prone rats.

For percentage of vehicle-corrected change in food intake at 4 wk, the statistical model also indicated a significant dose-response effect for amylin ($P < 0.05$ for quadratic effect) and a statistically significant synergy between amylin and leptin ($P < 0.05$; Fig. 2A and Table 1). As described for weight loss, synergy is demonstrated by a surface that becomes steeper (*i.e.* food intake reduction accelerates) as the doses of amylin and leptin are increased (Fig. 2B). As with the body weight data, the second-order interaction model fit to these data had a nonsignificant lack of fit, indicating that a higher-order polynomial model was unnecessary to adequately model the data. The food intake model had an r^2 value of 0.55.

Weight loss with amylin monotherapy was accompanied by dose-dependent decreases in leptin levels of up to 67% (Table 1). At the doses tested, leptin monotherapy did not significantly increase leptin levels above vehicle controls; however, the highest dose of leptin (125 µg/kg·d) given in combination with amylin clearly restored plasma leptin to a similar level as vehicle controls. Amylin treatment dose-dependently increased plasma amylin levels in the monotherapy and combination-therapy groups (Table 1).

Effects of amylin/leptin combination treatment compared with caloric restriction

Food intake, body weight, and body composition. To ascertain whether amylin/leptin administration exerts effects independently of those occurring in response to its anorexigenic properties, mechanistic endpoints in DIO rats administered the highest dose combination tested [amylin (50 µg/kg·d) plus leptin (125 µg/kg·d)] were compared with vehicle-treated rats that were matched for caloric intake (*i.e.* PF). Consistent with the effects observed in the initial experiments (RSM) with DIO-prone rats, amylin/leptin elicited robust decreases in food intake (Fig. 3A) and body weight (Fig. 3B) when administered to outbred DIO rats. After 26 d, cumulative food intake was reduced by 35% in amylin/leptin-treated animals compared with vehicle (288.8 ± 13.6 *vs.* 443.0 ± 7.0 g; $P < 0.01$); there was no difference in cumulative food intake between amylin/leptin-treated animals and PF controls (276.1 ± 0.3 g). Hypophagia (daily energy intake, expressed as kilojoules per gram body weight) in amylin/leptin-treated rats was most prominent during the first 10 d of the study, after which energy intake returned to, but did not exceed, that of vehicle controls (Fig. 3A).

Although amylin/leptin-treated rats and their PF controls exhibited similar weight loss, the composition of weight loss was notably different. Amylin/leptin-treated rats exhibited a 2-fold greater reduction in adiposity compared with PF rats (Fig. 3C). Histochemical visualization of retroperitoneal white adipose tissue showed modestly reduced adipocyte size in fat from PF animals, whereas adipocytes of amylin/

TABLE 1. Weight loss, inhibition in food intake, and analysis of plasma leptin and amylin levels after 4 wk treatment

Treatment group pump 1/pump 2 (μg/kg·d)	Weight loss (%; vehicle corrected)	Cumulative intake (% inhibition)	Plasma leptin (ng/ml)	Plasma amylin (pM)
Vehicle/vehicle	0 ± 1.5	0 ± 3.0	15.3 ± 4.4	7.5 ± 1.1
Vehicle/amylin (10)	-4.9 ± 1.0	−8.4 ± 2.8	8.9 ± 1.7	55.0 ± 8.9
Vehicle/amylin (50)	-6.9 ± 1.4	−14.0 ± 2.3	5.0 ± 1.3	386.4 ± 182.3
Vehicle/leptin (5)	-0.2 ± 2.2	−2.9 ± 4.3	12.4 ± 2.5	7.7 ± 1.2
Vehicle/leptin (25)	0 ± 1.0	−0.2 ± 1.7	13.3 ± 0.5	7.0 ± 1.0
Vehicle/leptin (125)	-2.3 ± 2.4	1.0 ± 4.1	17.5 ± 4.6	5.2 ± 0.8
Amylin (10)/leptin (5)	-4.8 ± 1.6	0.3 ± 5.3	11.6 ± 3.5	34.5 ± 6.8
Amylin (10)/leptin (25)	-1.5 ± 1.9	−8.5 ± 2.2	13.3 ± 1.1	42.9 ± 5.5
Amylin (10)/leptin (125)	-4.3 ± 1.1	−7.4 ± 2.1	28.4 ± 3.4	68.0 ± 8.9
Amylin (50)/leptin (5)	-8.1 ± 1.6	−13.1 ± 2.8	5.4 ± 1.1	357.0 ± 53.6
Amylin (50)/leptin (25)	-9.6 ± 0.4	−16.4 ± 2.1	5.7 ± 0.8	296.8 ± 37.5
Amylin (50)/leptin (125)	-14.8 ± 3.2	−24.6 ± 3.1	18.6 ± 3.7	283.4 ± 61.7

leptin-treated rats were considerably smaller, with portions within adipose tissue depots appearing almost completely devoid of lipid and more vascularized in appearance (Fig. 3D). Despite the marked weight loss, amylin/leptin treatment was also associated with a small but significant increase in percent lean mass relative to vehicle and PF, indicating a preservation of nonadipose tissue (Fig. 3C).

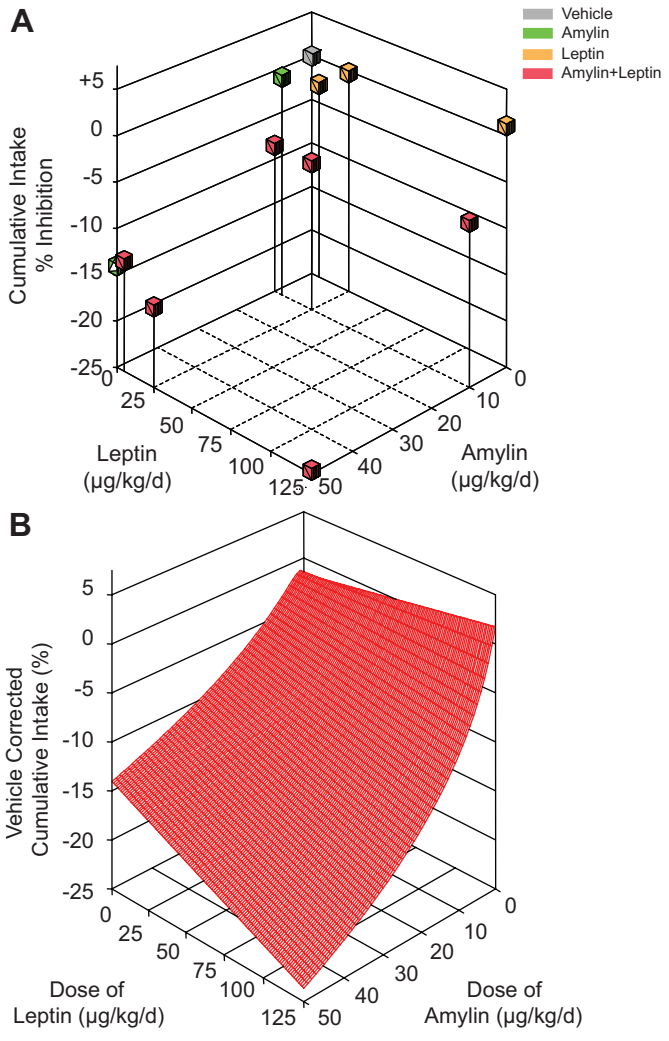

FIG. 2. A, Effects of amylin, leptin, and amylin/leptin on food intake; B, predicted response surface after 28 d exposure in DIO-prone rats.

Indirect calorimetry. To assess the effect of amylin/leptin- and PF-induced weight loss on energy metabolism, indirect calorimetry was performed during the first week (during active weight loss) and third week (when weight loss had stabilized) of treatment. Weight loss in PF rats was clearly accompanied by an expected counterregulatory decline in oxygen consumption (VO_2, a marker of metabolic rate) relative to vehicle (Fig. 4, A and B), an observation consistent with previous analyses of energy restriction effects on metabolism in rodents (19, 20). In contrast, amylin/leptin-treated rats consistently maintained VO_2 relative to vehicle controls throughout the study (Fig. 4, A and B). Furthermore, uncoupling protein-1 (*Ucp1*) mRNA levels (a molecular determinant of metabolic rate) (21) tended to be reduced in brown adipose tissue from PF rats but less so in amylin/leptin-treated rats (Fig. 4E). Because physical activity can be a significant component of total energy expenditure (22, 23), activity levels of vehicle, amylin/leptin, and PF rats were measured during the first week of treatment. No difference in pattern or amount of activity was observed between any of the groups (Fig. 4F).

The respiratory exchange ratio [RER; ratio of CO_2 production to O_2 (VCO_2:VO_2)] indicates the relative contribution of fat and carbohydrate oxidation to overall metabolism. During the first week, RER was significantly decreased in both amylin/leptin-treated and PF rats relative to vehicle controls, indicating increased utilization of fat as the fuel source during the initial period of active weight loss (Fig. 4C). However, during the period in which body weight had stabilized, a shift toward carbohydrate utilization was evident in PF, but not amylin/leptin-treated, rats. This discrepancy was particularly apparent at the onset of the dark phase when the animals were presented with food (Fig. 4D). Thus, in the face of chronic negative energy balance, amylin/leptin administration prevented the compensatory decline in metabolic rate and fat oxidation that was evident with PF.

Plasma glucose and lipid concentrations. To assess whether amylin/leptin-induced weight loss was associated with improvements in glucose and lipid homeostasis, fasting glucose was measured on d 14, and both glucose and triglycerides were

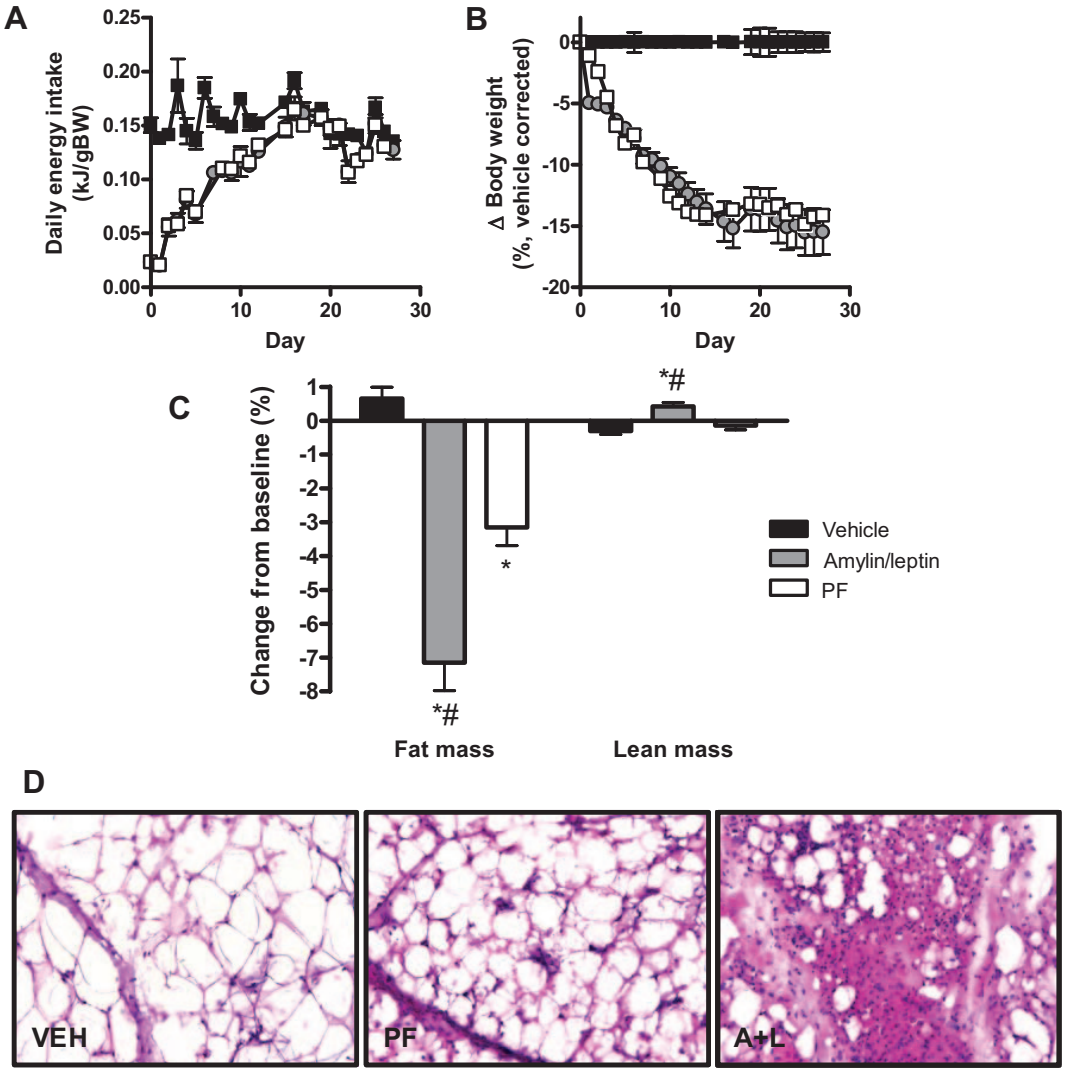

FIG. 3. Effect of amylin/leptin combination treatment on food intake, body weight, and body composition. A, Daily energy intake relative to body weight for DIO rats undergoing 28 d infusion of vehicle, amylin/leptin, or vehicle but matched for food intake of the amylin/leptin-treated rats (PF); B, change in body weight from baseline at the end of the study expressed as vehicle-corrected percentage weight loss; C, body composition analysis showing change in percent fat and lean mass of each treatment group; D, representative image of hematoxylin and eosin stain of retroperitoneal white adipose tissue from vehicle, PF, and amylin/leptin-treated rats (×20). BW, Body weight. *, $P < 0.05$ *vs.* vehicle; #, $P < 0.05$ *vs.* PF.

measured on d 28. On d 14, fasting blood glucose was significantly lower in amylin/leptin-treated rats (72 ± 7 mg/dl) compared with vehicle (110 ± 6 mg/dl; $P < 0.05$) and PF (91 ± 3 mg/dl; $P < 0.05$, data not shown). Fasting insulin results are not presented because plasma insulin levels in amylin/leptin-treated rats were below the sensitivity limit of the assay (0.2 ng/ml). On d 28, plasma triglyceride levels were significantly reduced in amylin/leptin and PF rats compared with vehicle; however, only amylin/leptin rats exhibited reduced total and high-density lipoprotein cholesterol (Table 2). There was no difference in glucose levels between groups on d 28.

Hepatic gene expression. To investigate potential underlying molecular mechanisms of amylin/leptin-mediated changes in metabolism and fat loss, the expression of select hepatic genes involved in the regulation of lipid metabolism were compared in vehicle- and amylin/leptin-treated animals and PF controls. Mean mRNA levels of stearoyl-coenzyme A desaturase-1 (*Scd1*), a leptin-sensitive mediator of fatty acid biosynthesis, was significantly reduced in amylin/leptin-treated rats (compared with vehicle controls) and tended to be reduced by PF ($P = 0.09$; Fig. 5A). Mean mRNA levels of another key lipogenic enzyme, fatty acid synthase (*Fasn*), was significantly reduced in amylin/leptin-treated rats relative to both vehicle and PF groups (Fig. 5B), whereas expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase-1 (*Pck1*) was significantly increased by amylin/leptin treatment relative to vehicle and PF groups (Fig. 5C).

Discussion

Amylin-induced weight loss in DIO rats has previously been shown to be fat specific, with relative preservation of

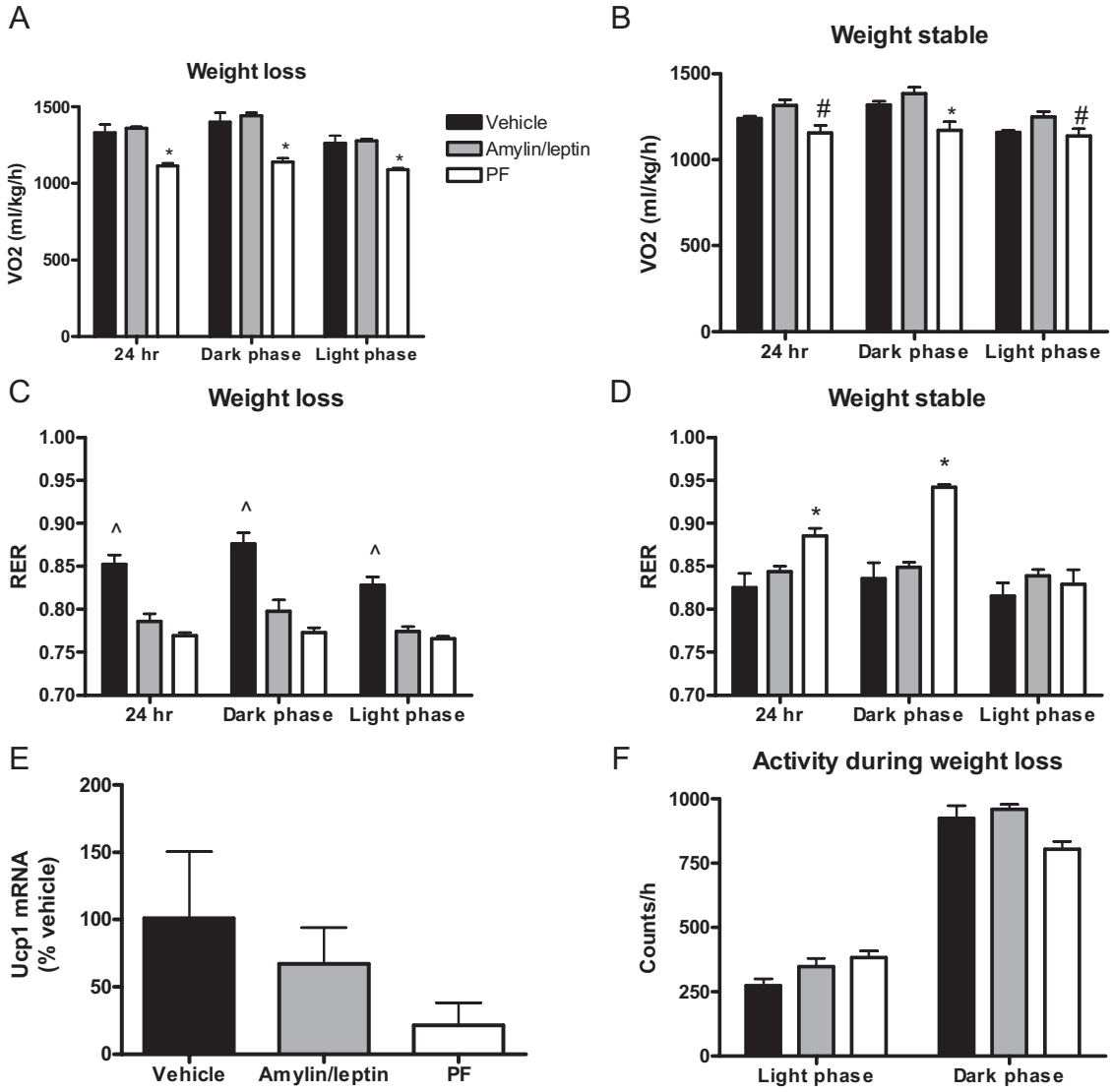

FIG. 4. Amylin/leptin combination treatment preserves metabolic rate and alters substrate preference during weight loss. A and B, Mean 24-h oxygen consumption (VO_2) of DIO rats undergoing 28 d infusion of vehicle, amylin/leptin, or PF rats during the first week of treatment when significant weight loss was occurring (A) and during the third week of treatment when weight loss had stabilized (B); 24-h RER data during the first (C) and third (D) week of treatment for the same animals; E, brown adipose tissue uncoupling protein-1 (*Ucp1*) mRNA levels; F, total 24-h physical activity data expressed as mean activity (counts of light beams broken in x- and y-axis per hour) for light and dark phases during the first week of treatment. *, $P < 0.05$ *vs.* vehicle and amylin/leptin groups; #, $P < 0.05$ *vs.* amylin/leptin group; ^, $P < 0.05$ *vs.* amylin/leptin and PF groups.

lean mass, associated with increased proopiomelanocortin gene expression within the hypothalamic arcuate nucleus and not accompanied by a compensatory decline in metabolic rate (12). These effects resemble those observed with leptin administration to leptin-sensitive animals and suggest that amylin may help restore or even increase endogenous leptin sensitivity. Pharmacological studies examining the weight loss effect of combined amylin and leptin agonism in DIO rats and overweight/obese humans have provided additional evidence that amylin agonism at least partially restores responsiveness to the antiobesity effect of leptin (17). The present preclinical findings provide important additional insights. First, our results constitute a formal, statistical demonstration of amylin/leptin synergy to decrease food intake and body weight in DIO rats. Second, synergistic body weight loss was durable over a 28-d period and obtained at considerably lower doses than previously tested. Lastly, whereas reduced caloric intake accounted for amylin/leptin-mediated weight loss, we provide evidence that the observed preferential reduction in adiposity with amylin/leptin treat-

TABLE 2. Plasma parameters at termination (d 28) of vehicle controls, amylin/leptin-treated, and chronically PF rats

Treatment group	Glucose (mg/dl)	Triglyceride (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)
Vehicle	129 ± 3	42 ± 6	97 ± 5	27 ± 1
Amylin/leptin	110 ± 10	15 ± 4[a]	79 ± 5[a]	22 ± 1[a]
PF	125 ± 6	27 ± 2[a]	95 ± 4	27 ± 1

Animals were fasted overnight before being killed and collection of plasma. HDL, High-density lipoprotein cholesterol.

[a] $P < 0.05$ *vs.* vehicle within parameter.

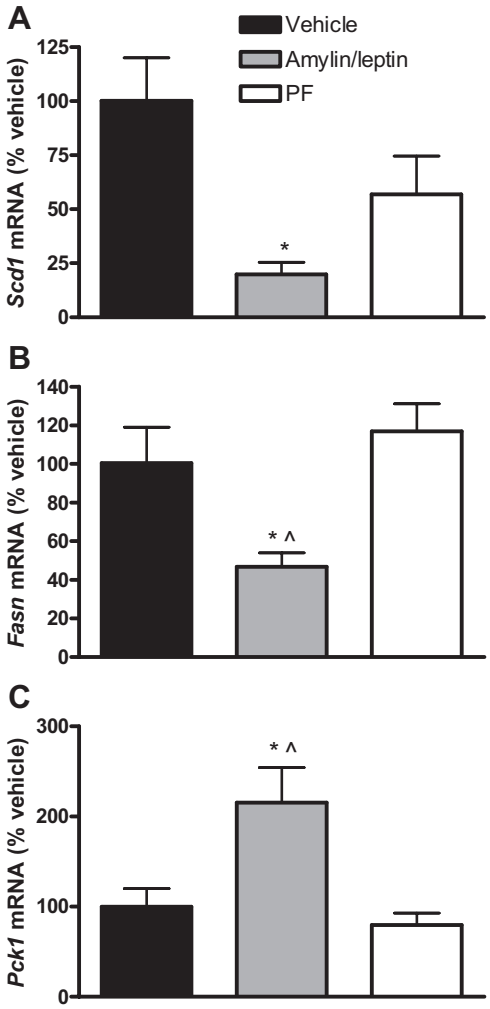

FIG. 5. Hepatic lipid metabolism is altered by peptide combination therapy. Expression of stearoyl-coenzyme A desaturase-1 (*Scd1*) (A), fatty acid synthase (*Fasn*) (B), and phosphenolpyruvate carboxykinase (*Pck1*) (C) in liver of DIO rats after infusion with vehicle or amylin/leptin or PF to the amylin/leptin group for 28 d. *, $P < 0.05$ compared with vehicle; ^, $P < 0.05$ compared with PF group.

ment may be due to changes in energy expenditure, substrate utilization, and/or changes in hepatic gene expression that occur independently of reduced calorie intake.

Amylin plus leptin interaction: RSM

The experiments conducted to support our RSM analyses confirmed our previous findings that the weight-reducing effects of amylin/leptin exceeded the predicted additive effects of single peptide administration. RSM is a powerful predictive statistical method for exploring responses to combination drug therapy and has been used for analyses of combination therapies for HIV, hypertension, and cancer (24–26). Our laboratory has previously used RSM to demonstrate synergy between amylin and peptide YY(3–36) for inhibition of food intake but not for weight loss (27). In the present study, RSM suggest a synergistic relationship between amylin and leptin for both food intake inhibition and weight loss, characterized by a downward steepening in the response surface that was most evident at the highest dose combination tested (amylin 50 μg/kg·d plus leptin 125 μg/kg·d). Monotherapy with amylin (50 μg/kg·d) increased plasma amylin levels by about 50-fold and reduced body weight and plasma leptin by 6–7 and 67%, respectively, after 28 d. Monotherapy with the relatively low dose of leptin (125 μg/kg·d) used in the present studies did not elicit meaningful weight loss or elevate leptin levels above vehicle controls. Plasma hyperleptinemia in this model has been achieved previously with infusion of higher concentrations of leptin without any appreciable reduction in body weight (17), suggesting that supraphysiological doses of leptin alone are insufficient to reduce body weight and inhibit food intake in DIO-prone rats. However, when the physiological dose of leptin used in the present study was combined with amylin, plasma leptin levels were restored to a level similar to that observed in vehicle controls, whereas body weight was reduced by 15% and food intake by 25%. These findings suggest that high pharmacological levels of leptin are not required for the observed synergism of these two neurohormones. This finding is consistent with clinical observations that leptin-associated weight control during periods of reduced caloric intake may result from the ability of physiological replacement doses of leptin to prevent weight loss counterregulatory mechanisms (3, 28) rather than induction of weight loss using high pharmacological doses.

Mechanisms underlying amylin/leptin-induced weight loss

To investigate metabolic mechanisms responsible for amylin/leptin-mediated weight loss that are independent of food intake inhibition, amylin/leptin-treated rats were compared with calorie-restricted controls (by PF). This comparison provided several important insights into the unique attributes of amylin/leptin-mediated weight loss. Amylin/leptin-treated rats exhibited a similar metabolic rate as *ad libitum* fed controls and significantly higher metabolic rate than PF rats. Maintenance of energy expenditure in the face of weight loss is a characteristic effect of exogenous hyperleptinemia in leptin-sensitive animals (13, 29). Similarly, chronic amylin treatment modestly increases, or at least maintains, energy expenditure in DIO rats (12) but has no effect on energy expenditure when administered acutely (30). Combination treatment with amylin and leptin markedly reduced caloric intake and body weight while preventing the compensatory reduction in energy expenditure observed after caloric restriction (PF rats).

Another unique and striking feature of amylin/leptin treatment became evident through body composition analysis. Despite similar weight loss in amylin/leptin and PF controls, amylin/leptin treatment induced an approximately 2-fold greater loss of fat mass compared with PF rats. Although a comprehensive assessment of fat cell size and number was not performed, visualization of white adipose tissue revealed that adipocytes from amylin/leptin-treated rats were generally smaller, with some areas virtually devoid of lipid compared with adipocytes from PF rats. Analysis of energy expenditure suggests that the greater fat loss after amylin/leptin treatment can be at least partly accounted for by the differences in metabolic rate between amylin/leptin

and PF animals during both active weight loss and weight maintenance periods. Consistent with other studies examining the effect of calorie restriction on energy expenditure (19, 20), chronically PF rats exhibited increased fat oxidation at the onset of reduced food intake. However, once weight loss had stabilized, substrate utilization in amylin/leptin-treated rats was similar to vehicle controls, whereas the RER of PF rats was significantly elevated compared with vehicle controls (indicating a shift to carbohydrate as the predominant fuel source). Thus, weight loss in amylin/leptin-treated rats was associated with preferential utilization of fat as the preferred substrate, likely contributing to the preferential reduction in adipose tissue mass.

An additional mechanism for enhanced fat loss with amylin/leptin treatment could be reduced lipid biosynthesis, possibly in conjunction with enhanced lipid oxidation. Expression of *Fasn*, a key mediator of fatty acid synthesis, was significantly reduced in amylin/leptin-treated rats compared with vehicle and PF rats, and similar patterns of expression were observed with *Scd1*. Both *Scd1* and *Fasn* are sensitive to exogenous leptin administration (31, 32), and low hepatic *Scd1* levels are also associated with increased energy expenditure, reduced adiposity, and improvements in hepatic steatosis and liver function (31, 33, 34). Suppression of *Fasn* and *Scd1* is indicative of leptin sensitivity, although chronic amylin administration alone may also reduce hepatic *Scd1* (35). Elevated *Pck1* expression could indicate enhanced hepatic gluconeogenesis in amylin/leptin-treated rats, also a known function of leptin action (36). Although corresponding protein levels or functional changes in hepatic lipid metabolism were not measured, the observed changes in hepatic gene expression are suggestive of amylin/leptin-induced reductions in body weight and adiposity mediated, at least partly, by marked inhibition of hepatic lipogenesis.

Conclusion

The complex, integrated, neurohormonal regulation of energy homeostasis is characterized by considerable redundancy of feeding pathways and potent counterregulatory responses. One rational, scientific approach to obesity drug development, therefore, is to combine peripheral hormones that target multiple sites of action and prevent/minimize metabolic counterregulatory adaptations triggered by weight loss. In a rodent model of obesity, the combined administration of the pancreatic hormone amylin (a short-term satiety signal) with the adipokine leptin (a long-term adiposity signal) appears to meet these criteria. Amylin/leptin treatment elicited synergistic effects on body weight and markedly reduced adiposity, yet prevented the decline in energy expenditure and shift toward carbohydrate utilization evoked by caloric restriction. Clinical evidence to support the concept of an integrated neurohormonal therapy for obesity was recently obtained in overweight/obese humans where combination treatment with the amylin analog pramlintide and the leptin analog metreleptin elicited considerably greater weight loss than did either agent alone (17). Thus, the synergistic effects of these peptides and their analogs may provide innovative treatment prospects for obesity.

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References

1. **Chen H, Roth JD, Schroeder BE, Weyer C** 2008 Role of islet-, gut-, and adipocyte-derived hormones in the central control of food intake and body weight: implications for an integrated neurohormonal approach to obesity pharmacotherapy. Curr Diabetes Rev 4:79–91
2. **Roth JD, Mack CM, Kesty NC, Parkes DG, Weyer C** 2008 Integrated neurohormonal approaches to the treatment of obesity: the amylin agonist pramlintide and its interaction with leptin and PYY signaling pathways. In: Bray G, ed. Handbook of obesity. 3rd ed. New York: Informa Healthcare; 341–361
3. **Rosenbaum M, Murphy EM, Heymsfield SB, Matthews DE, Leibel RL** 2002 Low dose leptin administration reverses effects of sustained weight-reduction on energy expenditure and circulating concentrations of thyroid hormones. J Clin Endocrinol Metab 87:2391–2394
4. **Lutz TA, Senn M, Althaus J, DelPrete E, Ehrensperger F, Scharrer E** 1998 Lesion of the area postrema nucleus of the solitary tract (AP/NTS) attenuates the anorectic effects of amylin and calcitonin gene-related peptide (CGRP) in rats. Peptides 19:309–317
5. **Lutz TA, Mollet A, Rushing PA, Riediger T, Scharrer E** 2001 The anorectic effect of a chronic peripheral infusion of amylin is abolished in area postrema/nucleus of the solitary tract (AP/NTS) lesioned rats. Int J Obes Relat Metab Disord 25:1005–1011
6. **Chapman I, Parker B, Doran S, Feinle-Bisset C, Wishart J, Lush CW, Chen K, LaCerte C, Burns C, McKay R, Weyer C, Horowitz M** 2007 Low-dose pramlintide reduced food intake and meal duration in healthy, normal weight subjects. Obesity (Silver Spring) 15:1179–1186
7. **Aronne L, Fujioka K, Aroda V, Chen K, Halseth A, Kesty NC, Burns C, Lush CW, Weyer C** 2007 Progressive reduction in body weight after treatment with the amylin analog pramlintide in obese subjects: a phase 2, randomized, placebo-controlled, dose-escalation study. J Clin Endocrinol Metab 92:2977–2983
8. **Wadden T, Klein S, Aronne L, Smith S, Halseth A, Kesty N, Burns C, Weyer C** 2006 Pramlintide treatment in obesity elicited progressive weight loss when used with a structured lifestyle intervention program: a randomized controlled trial. Obes Rev 7(Suppl 2):112–113 (Abstract PP0056)
9. **Smith SR, Blundell JE, Burns C, Ellero C, Schroeder BE, Kesty NC, Chen K, Halseth AE, Lush CW, Weyer C** 2007 Pramlintide treatment reduces 24-hour caloric intake and meal sizes, and improves control of eating in obese subjects: a 6-week translational research study. Am J Physiol Endocrinol Metab 293: E620–E627
10. **Lutz TA, Del Prete E, Scharrer E** 1994 Reduction of food intake in rats by intraperitoneal injection of low doses of amylin. Physiol Behav 55:891–895
11. **Rushing PA, Hagan MM, Seeley RJ, Lutz TA, Woods SC** 2000 Amylin: a novel action in the brain to reduce body weight. Endocrinology 141:850–853
12. **Roth JD, Hughes H, Kendall E, Baron AD, Anderson CM** 2006 Anti-obesity effects of the β-cell hormone amylin in diet induced obese rats: effects on food intake, body weight, composition, energy expenditure and gene expression. Endocrinology 147:5855–5864
13. **Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM** 1995 Weight-reducing effects of the plasma protein encoded by the obese gene. Science 269:543–546
14. **Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, Friedman JM** 1997 Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. Proc Natl Acad Sci USA 94:8878–8883
15. **Heymsfield SB, Greenberg AS, Fujioka K, Dixon RM, Kushner R, Hunt T, Lubina JA, Patane J, Self B, Hunt P, McCamish M** 1999 Recombinant leptin for weight loss in obese and lean adults: a randomized, controlled, dose-escalation trial. JAMA 282:1568–1575
16. **Farooqi IS** 2002 Leptin and the onset of puberty: insights from rodent and human genetics. Semin Reprod Med 20:139–144
17. **Roth JD, Roland BL, Cole RL, Trevaskis JL, Weyer C, Koda J, Anderson CM, Parkes DG, Baron AD** 2008 Leptin responsiveness restored by amylin agonism in diet-induced obesity: evidence from nonclinical and clinical studies. Proc Natl Acad Sci USA 105:7257–7262
18. **Levin BE, Dunn-Meynell AA, Balkan B, Keesey RE** 1997 Selective breeding

for diet-induced obesity and resistance in Sprague-Dawley rats. Am J Physiol 273:R725–R730
19. **Even PC, Nicolaidis S** 1993 Adaptive changes in energy expenditure during mild and severe feed restriction in the rat. Br J Nutr 70:421–431
20. **Forsum E, Hillman PE, Nesheim MC** 1981 Effect of energy restriction on total heat production, basal metabolic rate, and specific dynamic action of food in rats. J Nutr 111:1691–1697
21. **Jacobsson A, Stadler U, Glotzer MA, Kozak LP** 1985 Mitochondrial uncoupling protein from mouse brown fat. Molecular cloning, genetic mapping, and mRNA expression. J Biol Chem 260:16250–16254
22. **Leibel RL, Rosenbaum M, Hirsch J** 1995 Changes in energy expenditure resulting from altered body weight. N Engl J Med 332:621–628
23. **Weyer C, Pratley RE, Salbe AD, Bogardus C, Ravussin E, Tataranni PA** 2000 Energy expenditure, fat oxidation, and body weight regulation: a study of metabolic adaptation to long-term weight change. J Clin Endocrinol Metab 85:1087–1094
24. **Patick AK, Boritzki TJ, Bloom LA** 1997 Activities of the human immunodeficiency virus type 1 (HIV-1) protease inhibitor nelfinavir mesylate in combination with reverse transcriptase and protease inhibitors against acute HIV-1 infection in vitro. Antimicrob Agents Chemother 41:2159–2164
25. **Pool JL, Cushman WC, Saini RK, Nwachuku CE, Battikha JP** 1997 Use of the factorial design and quadratic response surface models to evaluate the fosinopril and hydrochlorothiazide combination therapy in hypertension. Am J Hypertens 10:117–123
26. **Carter Jr WH, Wampler GL** 1986 Review of the application of response surface methodology in the combination therapy of cancer. Cancer Treat Rep 70:133–140
27. **Roth J, Coffey T, Jodka C, Maier H, Athanacio J, Mack C, Weyer C, Parkes D** 2007 Combination therapy with amylin and PYY[3–36] in obese rodents: anorexigenic synergy and weight loss additivity. Endocrinology 148:6054–6061
28. **Rosenbaum M, Goldsmith R, Bloomfield D, Magnano A, Weimer L, Heymsfield S, Gallagher D, Mayer L, Murphy E, Leibel RL** 2005 Low-dose leptin reverses skeletal muscle, autonomic, and neuroendocrine adaptations to maintenance of reduced weight. J Clin Invest 115:3579–3586
29. **Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F** 1995 Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269:540–543
30. **Wielinga PY, Alder B, Lutz TA** 2007 The acute effect of amylin and salmon calcitonin on energy expenditure. Physiol Behav 91:212–217
31. **Cohen P, Miyazaki M, Socci ND, Hagge-Greenberg A, Liedtke W, Soukas AA, Sharma R, Hudgins LC, Ntambi JM, Friedman JM** 2002 Role for stearoyl-CoA desaturase-1 in leptin-mediated weight loss. Science 297:240–243
32. **Liang CP, Tall AR** 2001 Transcriptional profiling reveals global defects in energy metabolism, lipoprotein, and bile acid synthesis and transport with reversal by leptin treatment in ob/ob mouse liver. J Biol Chem 276:49066–49076
33. **Ntambi JM, Miyazaki M, Stoehr JP, Lan H, Kendziorski CM, Yandell BS, Song Y, Cohen P, Friedman JM, Attie AD** 2002 Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. Proc Natl Acad Sci USA 99:11482–11486
34. **Lee SH, Dobrzyn A, Dobrzyn P, Rahman SM, Miyazaki M, Ntambi JM** 2004 Lack of stearoyl-CoA desaturase 1 upregulates basal thermogenesis but causes hypothermia in a cold environment. J Lipid Res 45:1674–1682
35. **Roth JD, Hughes H, Coffey T, Maier H, Trevaskis JL, Anderson CM** 2007 Effects of prior or concurrent food restriction on amylin-induced changes in body weight and body composition in high-fat-fed female rats. Am J Physiol Endocrinol Metab 293:E1112–E1117
36. **Gutierrez-Juarez R, Obici S, Rossetti L** 2004 Melanocortin-independent effects of leptin on hepatic glucose fluxes. J Biol Chem 279:49704–49715

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