Cerulenin Mimics Effects of Leptin on Metabolic Rate, Food Intake, and Body Weight Independent of the Melanocortin System, but Unlike Leptin, Cerulenin Fails to Block Neuroendocrine Effects of Fasting

Hideo Makimura,[1] Tooru M. Mizuno,[1] Xue-Jun Yang,[1] Jeff Silverstein,[2] Joe Beasley,[2] and Charles V. Mobbs[1]

Cerulenin and a related compound, C75, have recently been reported to reduce food intake and body weight independent of leptin through a mechanism hypothesized, like leptin, to involve hypothalamic nutrition-sensitive neurons. To assess whether these inhibitors act through mechanisms similar to mechanisms engaged by leptin, *ob/ob* and A^y (agouti) mice, as well as fed and fasted wild-type mice, were treated with cerulenin. Like leptin, cerulenin reduced body weight and food intake and increased metabolic rate in *ob/ob* mice, and cerulenin produced the same effects in wild-type mice, whereas lithium chloride, at doses that produce conditioned taste aversion, reduced metabolic rate. However, in contrast to leptin, cerulenin did not prevent effects of fasting on plasma corticosterone or hypothalamic levels of neuropeptide Y, agouti-related peptide, pro-opiomelanocortin, or cocaine- and amphetamine-related peptide mRNA. Also, in contrast to leptin, cerulenin was highly effective to reduce body weight in A^y mice, in which obesity is caused by blockade of the melanocortin receptor. These data demonstrate that cerulenin produces metabolic effects similar to effects of leptin, but through mechanisms that are independent of, or down-stream from, both leptin and melanocortin receptors. *Diabetes* 50:733–739, 2001

It has recently been reported that cerulenin and a related compound, C75, robustly and reversibly reduce food intake and body weight with no detectable toxicity (1). Although both compounds inhibit the activity of fatty acid synthase (FAS) (2), cerulenin may also block protein acylation (3), and it has not been conclusively proven that the catabolic effects of these compounds are mediated through an inhibition of FAS. Nevertheless, the metabolic effects of these compounds were hypothesized to involve nutrient-sensitive hypothalamic neurons, possibly by the enhancement of hypothalamic levels of malonyl CoA through a pancreatic β-cell–like mechanism (1). In particular, Loftus et al. (1) suggested that these compounds might, like leptin, act in part by blocking the effects of fasting on hypothalamic gene expression, especially expression of hypothalamic neuropeptide Y (NPY). Because the weight-reducing effects of C75 were observed in leptin-deficient *ob/ob* mice (1), an obvious hypothesis is that these compounds act as leptin agonists to mimic effects of leptin (i.e., to block the effects of fasting on NPY expression). Three salient properties of the action of leptin are as follows: *1*) leptin increases metabolic rate in *ob/ob* and fasted wild-type, but not fed wild-type mice (4–6); *2*) leptin blocks numerous endocrine and hypothalamic effects of fasting (7,8); and *3*) A^y (agouti) mice are highly resistant to the effects of leptin (9,10), consistent with extensive data suggesting that the melanocortin system plays a key role in mediating the effects of leptin (11–16). To assess whether cerulenin may act as a leptin agonist, the present study addressed whether cerulenin increases metabolic rate, blocks effects of fasting on corticosterone and hypothalamic gene expression, and is effective to reduce body weight and food intake in A^y mice.

RESEARCH DESIGN AND METHODS

Male *ob/ob* and A^y (agouti) mice (C57BL/6J background) and their wild-type littermates, as well as CBA/J mice, were obtained at 2 months of age from the Jackson Laboratory (Bar Harbor, ME). Mice were individually housed with free access to food and water (except when otherwise specified) under a 12:12-h light-dark cycle (lights on at 7:00 A.M.). All studies were approved by the appropriate Institutional Animal Review Board.

To assess effects on metabolic rate, *ob/ob* or wild-type mice were injected with cerulenin (Sigma) (30 mg/kg i.p. every 12 h) or vehicle (20% DMSO in RPMI medium) for 4 days (n = 5–8/group). Body weight and food intake were determined daily. In the afternoon of the fourth day of injections, mice were placed into metabolic cages (Accuscan, Columbus, OH) for 4 h to determine metabolic rate and activity. Metabolic rate was determined using indirect calorimetry. Air from each cage was sampled once every 5 min; oxygen and carbon dioxide concentrations were independently determined for each sample of air. From these concentrations, the change in concentration of each gas (expressed as a percentage [$\%O_2$ and $\%CO_2$]) was assessed over each 5-min interval. From the change of each gas, VO_2 and VCO_2 were calculated as the volume of gas consumed and produced, respectively, per 5-min period, normalized to body weight (expressed as milliliters of gas per kilogram per minute). Heat production was calculated from the respiratory exchange ratio (RER) (RER = VCO_2 to VO_2) by the following formula: heat (calories/hour) = [(4.33 + 0.67 × RER) × VO_2 × (body weight) × 60]. Thus, heat, $\%O_2$, and $\%CO_2$ reflect total metabolic activity, whereas VCO_2 and VO_2 reflect metabolic activity per gram body weight. Physical activity was reflected by the breaking of infrared light beams that were set up in a two-dimensional array of 16 beams,

From the Departments of [1]Geriatrics and [2]Anesthesia, the Fishberg Center for Neurobiology, Neurobiology of Aging Laboratories, Mount Sinai School of Medicine, New York, New York.

Address correspondence and reprint requests to Charles V. Mobbs, Neurobiology of Aging Laboratories, Box 1639, Mt. Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. E-mail: mobbsc@alum.mit.edu.

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AGRP, agouti-related peptide; ANOVA, analysis of variance; CART, cocaine- and amphetamine-regulated peptide; FAS, fatty acid synthase; NPY, neuropeptide Y; POMC, pro-opiomelanocortin; RER, respiratory exchange ratio.

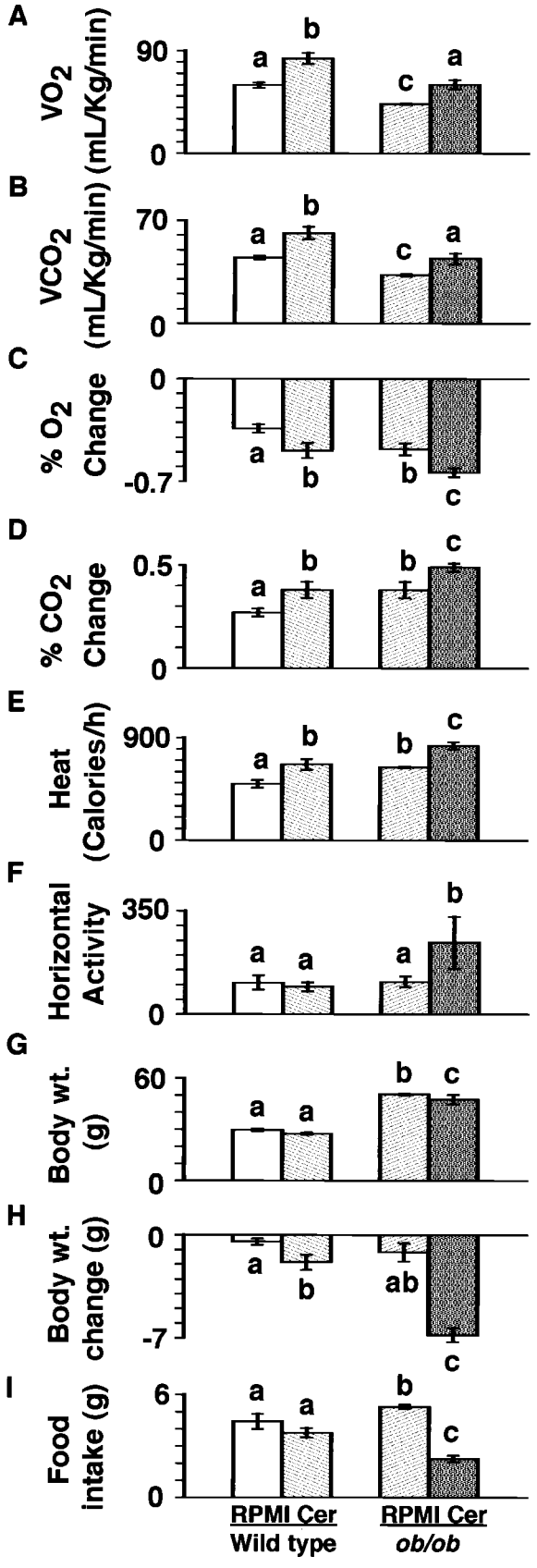

FIG. 1. Effect of cerulenin (Cer) or vehicle injections (RPMI) for 4 days on Vo_2 (volume of O_2 consumed per minute per kilogram body weight) (*A*), Vco_2 (volume of CO_2 produced per minute per kilogram body weight) (*B*), O_2 consumption (expressed as change in percentage per 5-min period) (*C*); CO_2 production (expressed as change in percentage per 5-min period) (*D*); heat production (absolute calories per hour, not normalized to body weight) (*E*); horizontal activity (number of beam breaks due to horizontal movement per 5-min period) (*F*); body weight (grams per 4 days) (*G*); body weight change (*H*); and food intake (grams per day) (*I*) in wild-type or *ob/ob* mice. Data are expressed as means ± SE, representing average values for the last 3 of 4 h in the metabolic cage. Groups with different letters are statistically different ($P < 0.05$ by ANOVA followed by Tukey-Kramer post hoc comparisons).

spaced 1 cm apart, with 8 beams projecting front-to-back and 8 beams projecting side-to-side. Horizontal activity was calculated as the number of beam breaks reflecting horizontal movement (consecutive positions) per 5-min period. Therefore, this parameter only reflected horizontal translation, not repetitive beam breaks that would more likely reflect stereotypic movement, such as scratching or grooming. To assess whether effects of cerulenin were due to conditioned taste aversion, a second set of C57BL/6J mice were injected intraperitoneally twice daily with lithium chloride (3 mEq/kg body wt, a daily dose that produces profound conditioned taste aversion in C57BL/6J mice) (17) or vehicle (n = 6/group), and metabolic parameters were assessed for comparison with effects of cerulenin.

To assess whether cerulenin blocked the effects of fasting, male CBA/J mice were fasted for 48 h or fed ad libitum and injected twice daily with cerulenin or vehicle (n = 5–10/group) following the method used by Ahima et al. (7) to assess the role of leptin in mediating neuroendocrine effects of fasting. The mice were killed toward the end of the light period (between 5:00 P.M. and 7:00 P.M.) by decapitation. Brains to be analyzed using in situ hybridization were removed, blocked, frozen on slides using dry ice, and stored at −70°C until use. Blood was taken for the analysis of hormones, and epididymal white adipose tissue was removed and weighed. To assess the sensitivity of A^y mice, A^y and wild-type mice were injected twice daily for 7 days with cerulenin or vehicle; body weight and food intake were assessed daily (n = 5/group). To assess the effects of cerulenin on food intake of previously fasted mice, a separate group of mice fasted for 48 h (with or without cerulenin treatment, as previously described) or ad libitum fed controls (also with or without cerulenin treatment) were given access to sweet milk at lights-out at the end of the fasting period.

Blood chemistry. Glucose was measured by a Lifescan One-Touch II glucose meter (Johnson & Johnson, Mountain View, CA). Insulin and leptin were assayed by enzyme-linked immunosorbent assay with commercial kits (Crystal Chem, Chicago), and corticosterone was assayed by radioimmunoassay with a commercial kit (ICN Pharmaceuticals, Costa Mesa, CA).

In situ hybridization histochemistry. In situ hybridization was carried out as previously described (12,13). Coronal sections of 10-μm thickness were cut through the mouse hypothalamus. The sections were fixed in 3% paraformaldehyde in 0.1 mmol/l phosphate buffer (pH = 7.0) containing 0.1% diethyl pyrocarbonate, dehydrated, and stored at −20°C until use. Sections were sorted based on histology to ensure that the anterior-posterior levels were matched between different mouse brains. Production and labeling of pro-opiomelanocortin (POMC), NPY, and agouti-related peptide (AGRP) probes and hybridization were carried out as previously described (12,13). A template DNA for cocaine- and amphetamine-related peptide (CART) (273 bp) was generated by reverse transcriptase–polymerase chain reaction from mouse hypothalamic RNA with NH_2-terminal primer, 5′-CCTGAAGAAGCTCAAGAGTAAACGC-3′, and COOH-terminal primer, 5′-GGGGGAACGCAAACTTTATTG-3′. The total integrated densities of hybridization signals were determined by computerized densitometric scanning (MCID, St. Catherine's, Ontario, Canada).

Statistical analysis. Statistical analysis entailed a two-way ([wild-type/mutant or fasting/fed condition] × cerulenin/vehicle) analysis of variance (ANOVA) followed by, when indicated by appropriate P values ($P < 0.05$), a Tukey-Kramer post hoc test, using the JMP statistical package implemented on the Macintosh operating system. Effects of lithium chloride were assessed by Student's t test. A P value <0.05 was considered significant.

RESULTS

Effect of cerulenin on body weight, food intake, metabolic rate, and activity of *ob/ob* and wild-type mice. To assess whether cerulenin produces an effect on metabolic rate similar to the effect of leptin, cerulenin was injected intraperitoneally into ad libitum fed wild-type and *ob/ob* mice. As previously described, cerulenin produced a significant decrease in body weight (Fig. 1*G* and *H*) and food intake in *ob/ob* mice (Fig. 1*I*); cerulenin also significantly reduced body weight in wild-type mice, but the effect on food intake did not quite reach significance in

C75BL/6J wild-type mice (Fig. 1*I*). Furthermore, these effects of cerulenin were accompanied by an increase in O_2 consumption and CO_2 production, as reflected in V_{O_2} and V_{CO_2} in both *ob/ob* and wild-type mice (Fig. 1*A* and *B*). Specifically, V_{CO_2} and V_{O_2} were lower in *ob/ob* mice than in wild-type controls; cerulenin increased V_{O_2} and V_{CO_2} in *ob/ob* mice to wild-type levels, and cerulenin increased V_{O_2} and V_{CO_2} to even higher levels in wild-type controls (Fig. 1*A* and *B*).

Although V_{O_2} and V_{CO_2} are standard measures of metabolic activity, these parameters reflect O_2 consumption and CO_2 production normalized for body weight (see RESEARCH DESIGN AND METHODS). However, because cerulenin decreases body weight, it is conceivable that the increases in both V_{CO_2} and V_{O_2} were simply caused by a decrease in nonmetabolically active tissue, rather than an increase in metabolic rate of metabolically active tissue. To address this question, average changes in both O_2 and CO_2 were calculated for each group. By this analysis, cerulenin increased absolute O_2 consumption and CO_2 production in both wild-type and *ob/ob* mice. Interestingly, by this analysis, *ob/ob* mice actually exhibit elevated total O_2 consumption and CO_2 production, relative to wild-type mice, presumably because *ob/ob* mice possess greater metabolically active mass than wild-type mice (but are not proportionally as metabolically active). In any case, because cerulenin increased both total O_2 consumption and CO_2 production, the increase in V_{CO_2} and V_{O_2} in both wild-type and *ob/ob* mice is not caused by the reduction in body weight produced by cerulenin (Fig. 1*C* and *D*). This conclusion is consistent with the observation that cerulenin caused an increase in absolute heat production in both wild-type and *ob/ob* mice (Fig. 1*E*). Furthermore, the increase in metabolic rate does not appear to be due solely to increased activity. By several measures of activity, cerulenin did not increase activity in wild-type mice, although it did increase metabolic rate. However, like leptin, cerulenin increased activity in *ob/ob* mice (Fig. 1*F*).

To assess whether these metabolic effects of cerulenin were caused by conditioned taste aversion, a second group of wild-type C57BL/6J mice were injected intraperitoneally with lithium chloride at a dose that produced profound taste aversion (17). Lithium chloride reduced food intake and body weight to approximately the same extent as cerulenin (Fig. 2*B* and *C*), but lithium chloride reduced, rather than enhanced, metabolic activity, as indicated by total heat production (Fig. 2*A*).

Effects of cerulenin on hypothalamic and endocrine responses to fasting. To assess whether cerulenin blocks the effects of leptin on hypothalamic and neuroendocrine responses to fasting, cerulenin or vehicle was administered twice daily for 48 h to ad libitum fed or fasted mice; mice were killed 30 min after the fourth injection. Over this 2-day period, fasting produced a significant reduction in body weight (Fig. 3*A*), fat-pad size (Fig. 3*B*), plasma glucose (Fig. 3*D*), insulin (Fig. 3*E*), and leptin (Fig. 3*F*). In addition, fasting led to an increase in plasma corticosterone (Fig. 4*A*), hypothalamic AGRP mRNA (Fig. 4*B*), and hypothalamic NPY mRNA (Fig. 4*C*) in the arcuate nucleus and a decrease in hypothalamic POMC (Fig. 4*D*) and CART mRNA (Fig. 4*E*) in the peri-arcuate nuclear area. In fasted mice, cerulenin had no impact on any of these parameters

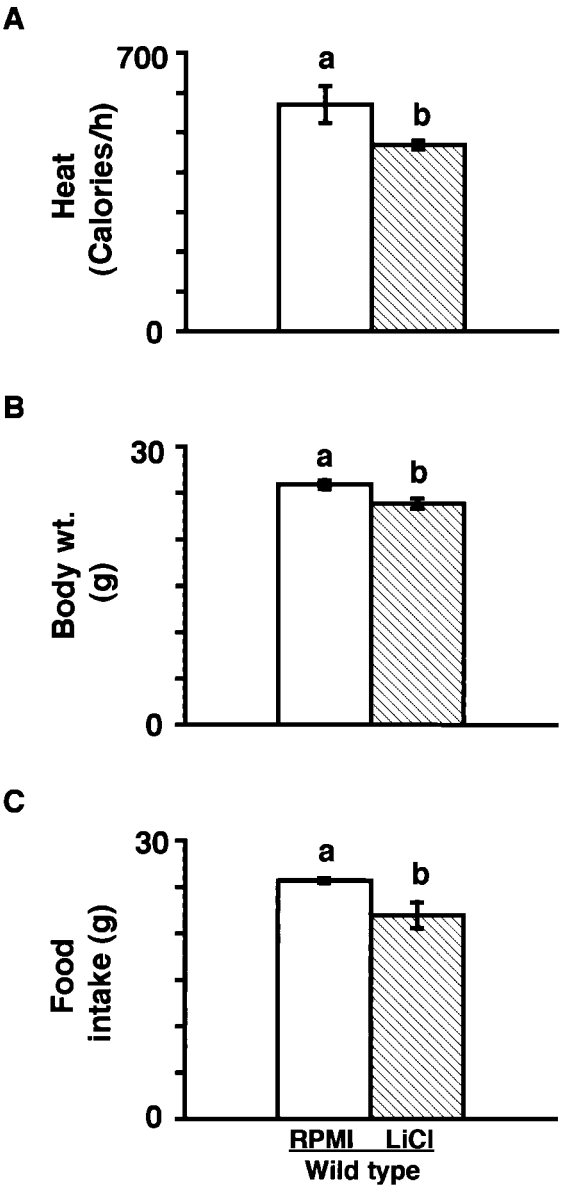

FIG. 2. Effect of lithium chloride (LiCl) or vehicle (RPMI) injection for 4 days on heat production (*A*); body weight (*B*); and food intake in wild-type C57BL/6J mice (*C*). Data are expressed as means ± SE, representing average values for the last 3 of 4 h in the metabolic cage. Groups with different letters are statistically different ($P < 0.05$ by Student's *t* test).

(Figs. 3 and 4); this demonstrates that cerulenin did not block effects of fasting on these parameters. Furthermore, cerulenin treatment partially mimicked many effects of fasting in ad libitum fed mice, leading to a decrease in body weight (Fig. 3*A*), fat-pad size (Fig. 3*B*), food intake (Fig. 3*C*), and (most important) an elevation in plasma corticosterone (Fig. 4*A*) and hypothalamic AGRP mRNA (Fig. 4*B*), with a nonsignificant trend in POMC mRNA (Fig. 4*D*). Corroborating the observations by Loftus et al. (1), cerulenin did not influence expression of hypothalamic NPY mRNA in ad libitum fed mice (Fig. 4*C*).

An interesting exception to the observation that leptin generally reverses neuroendocrine effects of fasting is that doses of leptin that reverse hormonal responses to fasting fail to reverse fasting-induced hyperphagia (7). This observation is particularly of interest, because it has been

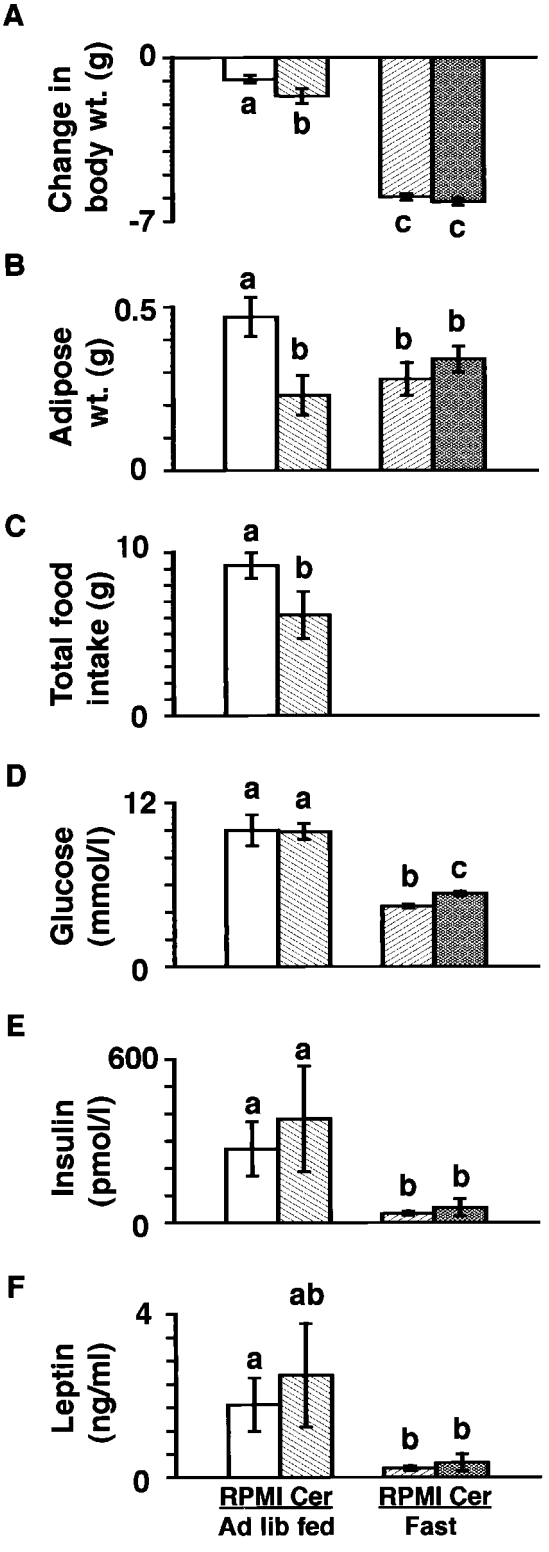

FIG. 3. Effect of cerulenin (Cer) or vehicle (RPMI) injection for 2 days in ad libitum fed or fasted mice on body weight (*A*); adipose weight (*B*); total food intake (*C*); plasma glucose (*D*); plasma insulin (*E*); and plasma leptin (*F*) in ad libitum fed and fasted CBA mice. Groups with different letters are statistically different ($P < 0.05$ by ANOVA followed by Tukey-Kramer post hoc comparisons).

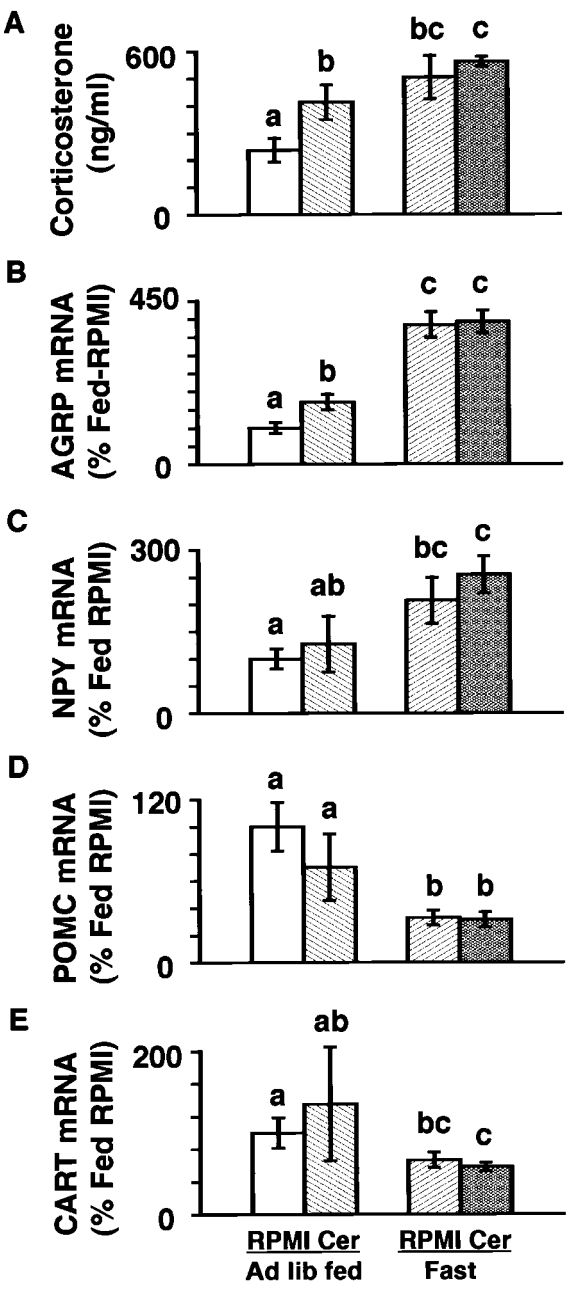

FIG. 4. Effect cerulenin (Cer) or vehicle (RMPI) injection for 2 days in ad libitum fed or fasted mice on plasma corticosterone (*A*); hypothalamic AGRP mRNA (*B*); hypothalamic NPY mRNA (*C*); hypothalamic POMC mRNA (*D*); and hypothalamic CART mRNA (*E*). Data are expressed as a percentage of mean vehicle-injected ad libitum fed levels ± SE. Groups with different letters are statistically different ($P < 0.05$ by ANOVA followed by Tukey-Kramer post hoc comparisons).

observed that agents thought to be physiological satiety agents, such as cholecystokinin, are less effective to reduce food intake in fasted individuals than in fed individuals, whereas nonphysiological anorexic substances, such as lithium chloride, reduce food intake at least as effectively in fasted individuals as in fed individuals (18). Therefore, we examined whether cerulenin would reduce consumption of a sweet-milk meal at lights out in mice previously fasted for 48 h and in mice fed ad libitum on a standard laboratory diet. As with leptin (7) and cholecystokinin (18), but in contrast to lithium chloride (18), when mice were fasted, cerulenin treatment did not significantly reduce milk consumption in previously fasted mice (cerulenin 91 ± 15% and vehicle-treated 100 ± 16%, expressed as percentage of vehicle-treated fasted controls), whereas, as expected, cerulenin treatment significantly reduced

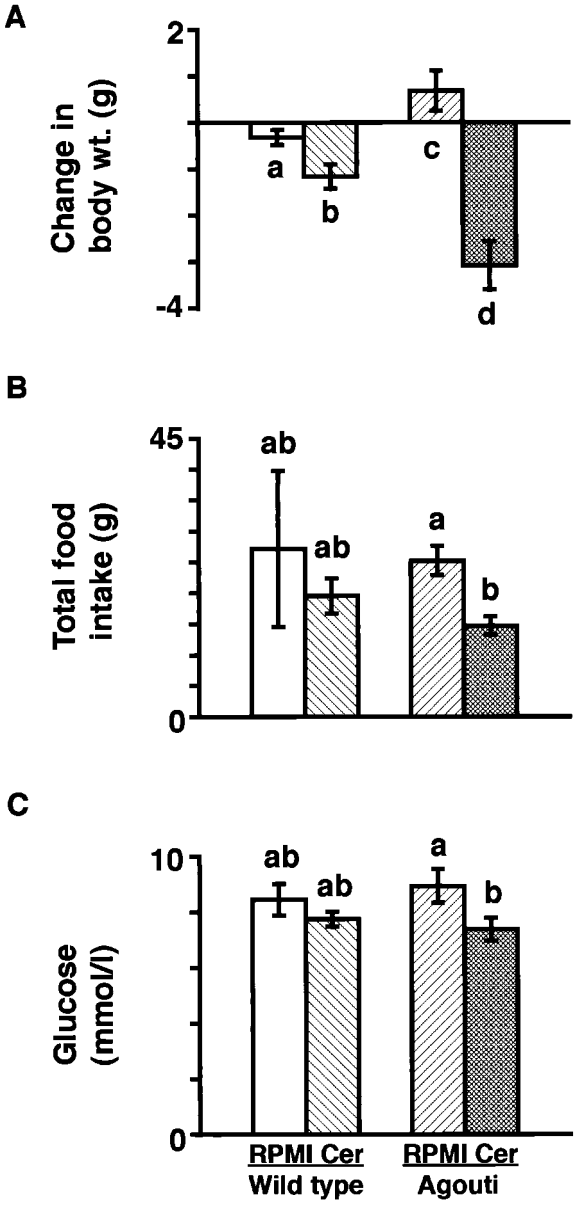

FIG. 5. Effect of cerulenin (Cer) or vehicle (RPMI) on body weight (*A*), food intake (*B*), and blood glucose (*C*) in wild-type and A^y mice. Data are expressed as means ± SE. Groups with different letters are statistically different ($P < 0.05$ by ANOVA followed by Tukey-Kramer post hoc comparisons).

consumption of sweet milk in ad libitum fed mice (cerulenin 65 ± 6% and vehicle-treated 100 ± 12%, expressed as a percentage of vehicle-injected controls).

Effects of cerulenin in A^y mice. The observation that cerulenin treatment did not influence hypothalamic NPY, POMC, or CART mRNA in fasted mice and actually led to an appropriate increase in AGRP mRNA in ad libitum fed mice, suggests that the effects of cerulenin are not mediated by increased activity of the melanocortin system. To definitively address this question, A^y and wild-type controls were treated with cerulenin for 1 week. As with *ob/ob* obese mice, A^y mice were actually more sensitive than wild-type mice to the effects of cerulenin to reduce body weight (Fig. 5*A*), food intake (Fig. 5*B*), and blood glucose (Fig. 5*C*).

DISCUSSION

The present study demonstrates that cerulenin is highly effective to reduce body weight, food intake, and fat pad size in the absence of leptin and in A^y agouti mice, which are obese because of impaired signaling of the melanocortin system. Furthermore, fasting-induced elevation of corticosterone and hypothalamic AGRP and NPY mRNA and reduction of hypothalamic POMC and CART mRNA were not attenuated by cerulenin treatment. Our results are completely consistent with the report that C75, a compound related to cerulenin and even more effective to reduce food intake and body weight, did not cause an increase in hypothalamic NPY mRNA (1). In contrast, treatment with leptin at doses that produce similar effects on body weight blocked the effects of fasting on corticosterone (7), as well as hypothalamic AGRP, NPY, POMC, and CART (10,19). Thus, these studies demonstrate that cerulenin does not act as either a leptin or a melanocortin agonist with respect to the parameters examined.

Nevertheless, although cerulenin does not act as a leptin agonist, cerulenin does mimic some effects of leptin to stimulate metabolic activity, as well as to reduce food intake and body weight (Fig. 1). Indeed, cerulenin may constitute a more potent stimulus to metabolic activity than leptin, because cerulenin stimulates metabolic activity in both wild-type ad libitum fed and *ob/ob* mice, whereas leptin fails to stimulate metabolic activity in ad libitum fed wild-type mice (20,21). The extent to which increased metabolic rate contributes to weight loss during cerulenin treatment remains unclear. At least in wild-type mice, the effects of cerulenin on metabolic activity (i.e., heat production) appear to be at least as great as the effects on food intake (which in C57BL/6J wild-type mice did not even achieve statistical significance). Furthermore, adipose weight in cerulenin-treated mice was reduced to the adipose weight of fasted mice (Fig. 3*B*), whereas the effects of cerulenin on food intake (Fig. 3*C*) and body weight change (Fig. 3*A*) were much less pronounced. Indeed, the effective food restriction was so mild that glucose, insulin, and leptin were not reduced at all by treatment with cerulenin; the maintenance of glucose and insulin probably accounts for the maintenance of leptin in the presence of reduced adiposity, because leptin levels are significantly regulated by circulating insulin and glucose (22). Taken together, these observations suggest that cerulenin causes a preferential loss of adipose tissue that is not highly correlated with food intake. Such a result is consistent with an important role for metabolic activity in mediating the effects of cerulenin on body weight, but this link has yet to be established.

The mechanism by which cerulenin increases metabolic activity and reduces adiposity and food intake remains unclear. Although cerulenin and C75 are both inhibitors of fatty acid synthase (2), it is not clear whether cerulenin acts by inhibiting fatty acid synthase or through other mechanisms. For example, cerulenin is reported to inhibit protein acylation (3), so it is possible that inhibition of protein acylation may mediate some effects of cerulenin. It is also possible that toxic effects may mediate some effects of cerulenin. One of the most obvious potential pharmacological effects would be the production of conditioned taste aversion. Although we have not yet been able to

reliably assess whether cerulenin produces conditioned taste aversion, we have shown that conditioned taste aversion is, in any case, unlikely to explain the metabolic effects of cerulenin, because lithium chloride, which does produce profound conditioned taste aversion (17), reduced, rather than enhanced, metabolic activity (Fig. 2*A*). Furthermore, chronic treatment with lithium chloride does not lead to a prolonged reduction in body weight or food intake (23), in contrast to persistent effects of cerulenin to decrease both parameters (1). Similarly, cerulenin increased or had no effect on horizontal activity (Fig. 1*F*), whereas toxic insults, especially those involving cytokines, reduce physical activity (24) and typically do not lead to prolonged weight loss or food intake (25). In addition, whereas cerulenin treatment led to a modest increase in plasma corticosterone in ad libitum fed mice, consistent with a mildly food-restricted state, cerulenin did not cause increased corticosterone in fasted mice, as would be expected if cerulenin were producing a nonspecific stress response. Of particular interest, cerulenin, like leptin (7) and cholecystokinin (18), but unlike lithium chloride (18), failed to block food intake in previously fasted mice; this behavior is thought to characterize agents that act through a physiological satiety mechanism (18). Altogether, the present data suggest that cerulenin reduces body weight through mechanisms involving both reduced food intake and elevated metabolic rate, not consistent with known effects of toxins, nonspecific stressors, or cytokines. Nevertheless, such effects cannot yet be ruled out completely.

Loftus et al. (1) reported that C75 reduced food intake when infused directly into the third ventricle of the brain, and the effect of C75 was blocked by infusing into the brain the compound 5-(tetradecyloxy)-2-furoic acid, which inhibits acetyl CoA carboxylase. Based on these data, the authors hypothesized that C75 acts by enhancing the levels of malonyl CoA in hypothalamic neurons. In turn, these authors suggested that these effects may be mediated through glucose-sensitive hypothalamic neurons, because these neurons appear to sense glucose through mechanisms similar to those activated by glucose in pancreatic β-cells (26), and extensive studies have implicated malonyl CoA as a key mediator of effects of glucose in pancreatic β-cells (27,28). Our previous results on the hypothalamic glucose sensing mechanism are specifically consistent with a key role for malonyl CoA, because we observed that, as in pancreatic β-cells, the production of cytoplasmic NADH is an essential signal in the hypothalamic glucose-sensing mechanism (26). Therefore, these data are consistent with recent studies demonstrating an essential role for the NADH shuttle mechanism in mediating effects of glucose on insulin secretion (29), because activation of this shuttle system would be expected to lead to the anaplerotic pathways that produce malonyl CoA (30). We have observed that similar mechanisms mediate effects of glucose on glucose-inhibited hypothalamic neurons (X-J.Y., L.M. Kow, and C.V.M., unpublished observations). Nevertheless, although an appealing hypothesis, it remains to be demonstrated whether fatty acid synthase or glucose-sensitive hypothalamic neurons mediate effects of cerulenin on body weight, food intake, and metabolic activity.

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