Low Plasma Leptin Levels Contribute to Diabetic Hyperphagia in Rats

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The adipocyte hormone leptin reduces food intake in normal animals. During uncontrolled type 1 diabetes, plasma leptin levels fall, whereas food intake increases. To test the hypothesis that low leptin levels contribute to diabetic hyperphagia, we investigated the effect on food intake of replacement of leptin at basal plasma concentrations for 7 days in Long-Evans rats with uncontrolled diabetes induced by streptozotocin (STZ). One group of STZ diabetic rats received saline (STZ + Sal) (n = 11), while the other group (STZ + Lep) (n = 15) received a subcutaneous infusion of recombinant rat leptin (100 µg · kg^{-1} · day^{-1}) via osmotic minipumps. A nondiabetic control group (Con) (n = 11) received saline only. In the STZ + Sal group, plasma leptin levels decreased by 75% ($P < 0.05$) from 2.4 ± 0.5 on the day before STZ/citrate buffer vehicle (Veh) injection (day 0) to 0.6 ± 0.2 ng/ml on day 7. In contrast, plasma leptin levels on days 3–7 were comparable to pretreatment values in both the STZ + Lep group (day 0: 2.6 ± 0.4 vs. day 7: 2.5 ± 0.3 ng/ml, NS) and the Con group (day 0: 3.8 ± 0.4 vs. day 7: 2.9 ± 1.0 ng/ml, NS). In the STZ + Sal group, daily food intake increased gradually to values 43% above basal by day 7 (day 0: 24 ± 2 to day 7: 33 ± 3 g, $P < 0.05$), whereas food intake did not increase in either the STZ + Lep group (day 0: 24 ± 1 vs. day 7: 21 ± 2 g, NS), or the Con group (day 0: 23 ± 1 vs. day 7: 23 ± 2 g). Plasma glucose levels exceeded nondiabetic control values (7.7 ± 0.2 mmol/l) in both diabetic groups, but were lower in the STZ + Lep group (17.2 ± 1.8 mmol/l) than in the STZ + Sal group (24.3 ± 1.1 mmol/l, $P < 0.05$). To determine if sensitivity to leptin-induced anorexia was affected by STZ treatment, a second experiment was performed in which the effect of intracerebroventricular leptin injection (at doses of 0.35, 1.0, or 3.5 µg) on food intake was measured 10 days after STZ or Veh treatment. Leptin suppressed both 4- and 24-h food intake in the two groups to an equal extent at every dose (by 15, 22, and 35%, respectively). These findings support the hypothesis that the effect of uncontrolled diabetes to lower leptin levels contributes to diabetic hyperphagia and that this effect is not due to altered leptin sensitivity. ***Diabetes*** **48:1275–1280, 1999**

The hypothesis that body fat stores are subject to homeostatic regulation proposes that humoral signals generated in proportion to adiposity act in the central nervous system (CNS) to reduce food intake and body weight (1,2). Insulin, secreted from the pancreatic β-cells, and leptin (3), secreted from adipocytes, are both implicated in this homeostatic control system. Both hormones are secreted in proportion to body fat stores (4,5) and recent energy intake (6–8), and receptors for both insulin and leptin are concentrated in hypothalamic areas that control food intake and energy expenditure (9,10). Moreover, administration of either hormone into the brain (11–14) causes sustained reductions of food intake and body weight, whereas both insulin deficiency (as occurs in uncontrolled diabetes) and leptin deficiency (as seen in *ob/ob* mice [3]) are associated with marked hyperphagia. Leptin secretion depends on insulin stimulation of the adipocyte (15–21), an effect that appears to be mediated in part via increased adipocyte glucose uptake (22). Thus, during uncontrolled insulin-deficient diabetes induced by streptozotocin (STZ), a marked decrease in plasma concentrations of both insulin and leptin occurs, and this effect precedes the onset of hyperphagia characteristic of the diabetic state (23). Moreover, 2 days of insulin treatment of diabetic animals partially restored plasma leptin levels and reduced diabetic hyperphagia by half (23).

Together, these findings suggest that diabetic hyperphagia may result not from weight loss, glycosuria, or other metabolic derangements of the diabetic state, but from the direct effects of decreased signaling within the brain by leptin, insulin, or both hormones. To investigate the role of leptin deficiency per se as a mediator of diabetic hyperphagia, we sought to determine whether maintenance of normal plasma leptin levels in animals with uncontrolled diabetes attenuates the hyperphagic response. Leptin was infused subcutaneously at a dose designed to maintain basal circulating leptin concentrations and thereby prevent the hypoleptinemic state characteristic of insulin-deficient diabetes. We also investigated whether differences in the sensitivity to leptin's anorexic effect contribute to the pathogenesis of diabetic hyperphagia, by determining whether STZ-induced diabetes alters the dose-response characteristics of the anorexic response to centrally administered leptin.

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Received for publication 10 November 1998 and accepted in revised form 11 March 1999.

P.J.H. has received grant support from Amgen.

CNS, central nervous system; Con, control; CSF, cerebrospinal fluid, ICV, intracerebroventricular; Lep, leptin; NPY, neuropeptide Y; Sal, saline; STZ, streptozotocin; Veh, citrate buffer vehicle.

RESEARCH DESIGN AND METHODS

Animals and experimental procedures. Experiments were performed on male Long-Evans rats that were housed individually in a temperature-controlled environment (22° C) with a 12:12-h light:dark cycle; the rats were obtained from the breeding colony maintained by the Department of Psychology at the University of Washington. All rats had ad libitum access to pelleted rat food (Harlan-Teklad, Madison, WI) and tap water. All procedures were performed in accordance with institutional guidelines of the Animal Care Committee at the University of Washington.

Experiment 1. One week before the experiment, rats (~250 g) were anesthetized with an intraperitoneal injection of ketamine:xylazine (60:8 mg/kg), and an indwelling catheter was inserted into the right jugular vein. The catheter was extended to the level of the right atrium, and the free end of the catheter was passed subcutaneously to the back of the neck where it was externalized and sutured in place. Animals were treated prophylactically with gentomycin (8 mg i.m.) and were allowed to recover to presurgical levels of body weight and daily food intake before each experiment.

On day 0, the rats were divided into three groups of equivalent mean body weight and daily food intake. Two groups received a subcutaneous injection (40 mg/kg) of freshly prepared STZ (Sigma, St. Louis, MO) in 0.5 mol citrate buffer (pH 4.5) on each of 2 consecutive days to induce diabetes. This regimen has been shown to produce type 1 diabetes (plasma glucose >20 mmol/l) in >95% of treated animals without inducing renal failure or hypoglycemia (24). Subsequently, under ketamine:xylazine anesthesia, one group (STZ + saline [Sal]) (n = 11) received a subcutaneous implanted osmotic minipump (Alza Scientific Products, Palo Alto, CA) that infused saline alone, while the other diabetic group (STZ + leptin [Lep]) (n = 15) received a subcutaneous implanted osmotic minipump containing recombinant rat leptin (Amgen, Thousand Oaks, CA) diluted in saline with an infusion rate of 100 $\mu g \cdot kg^{-1} \cdot day^{-1}$. The third group remained nondiabetic, receiving two consecutive daily subcutaneous injections of citrate vehicle (Veh) rather than STZ, and subcutaneous osmotic minipump containing saline (Con). Plasma samples, food intake, and body weight measurements were taken every morning for 1 week after the first STZ/Veh injection.

Blood samples (1 ml) were obtained at 0800 (normal nonfasting conditions 1 h after "lights on") and were placed into tubes containing EDTA (1 mg/ml). Plasma was separated by centrifugation and red blood cells were washed and resuspended in sterile saline and reinfused to prevent anemia. Plasma samples were kept in an ice bath during processing and then stored at –70° C until assayed for determination of insulin, leptin, and glucose concentrations. Plasma glucose was determined by the glucose oxidase method (Beckman Instruments, Brea, CA). Plasma insulin (25,26) and leptin levels (Linco, St. Louis, MO) (27) were determined by radioimmunoassay as previously described.

Experiment 2. Two weeks before the experiment, rats (~350 g) underwent stereotaxic cannulation of the third cerebral ventricle under ketamine:xylazine anesthesia as previously described (28). Animals were allowed to recover to presurgical levels of weight and food intake before the experiment was begun. Patency of the intracerebroventricular (ICV) infusion system and verification of cannula placement were confirmed by ICV injection of angiotension II (10 ng in 1 µl) 1 week after the surgical procedure. Animals that did not drink 5 ml of water within 60 min after treatment were not included in the experiment.

After cannulation, rats were separated into two groups of equivalent mean body weight and daily food intake. One group (n = 17) received a single dose of STZ (65 mg/kg) via a tail vein under light halothane anesthesia to induce insulin-deficient diabetes, while control animals (n = 13) received citrate buffer only (Veh). The two groups were subsequently maintained on their standard diet for 10 days, and food intake and body weights were monitored daily. After diabetic hyperphagia was established in the STZ group, rats were adapted to a schedule in which food was removed from the cage and the animals were weighed 3 h before the end of the light cycle. On experimental days, 45 min after the food was removed, each animal was injected with human leptin (0, 0.35, 1.0, or 3.5 µg) (Hoffmann-LaRoche, Nutley, NJ) in synthetic cerebrospinal fluid via the ICV cannula in a volume of 3.5 µl. Food was returned at "lights out," and food intake was measured over the next 4 h and again 24 h after the leptin injection. Each diabetic rat received each dose of leptin with the order counterbalanced across subjects using a Latin-square design, with no injections occurring on the 4 intervening days between consecutive experimental days.

Statistics. All data are expressed as means ± SE. The level of significance was $P < 0.05$. The data were analyzed for differences on the basis of group-by-group comparisons and for changes from intragroup baseline values using one-way analysis of variance. Statistical comparisons were made using the Statview II (Calabasas, CA) computer program. Fischer's protected least significant difference test for multiple comparisons was used post hoc when significant F ratios were obtained. Experiment 2 was analyzed using standard mixed-model analysis of variance (STZ versus Veh) for the between-subjects variable and dose (as the within-subjects variable) on the raw food intake data at 4 and 24 h, followed by Tukey's paired sample or independent t test, as appropriate.

RESULTS

Experiment 1. In the STZ + Sal group, plasma insulin (Fig. 1) fell significantly from 377 ± 104 (day 0) to 202 ± 27 pmol/l by day 3 ($P < 0.05$) and remained low thereafter (186 ± 45 pmol/l, day 7), and this was associated with an increase of plasma glucose concentrations (Fig. 1) from 8.0 ± 0.2 (day 0) to 19.4 ± 1.7 mmol/l ($P < 0.05$) by day 2 and was increased threefold over basal by the end of the experiment (24.3 ± 1.1 mmol/l, day 7). In the STZ + Lep group, plasma insulin concentrations fell comparably from 317 ± 36 (day 0) to 220 ± 28 pmol/l by day 3 ($P < 0.05$) and remained low for the duration of the experiment (201 ± 36 pmol/l, day 7). Hyperglycemia was evident by day 2 (13.9 ± 1.5 mmol/l, $P < 0.05$) and was increased by more than twofold over basal by day 7 (17.2 ± 1.8 mmol/l, $P < 0.05$) in the STZ + Lep group, but the degree of hyperglycemia was significantly lower than that achieved in the STZ + Sal group. In contrast, neither plasma insulin (378 ± 63 pmol/l, days 0–7) nor plasma glucose levels (7.7 ± 0.2 mmol/l) changed significantly over the course of the experiment in nondiabetic controls.

Plasma leptin levels (Fig. 2) fell significantly in the STZ + Sal group from 2.4 ± 0.5 (day 0) to 1.1 ± 0.3 ng/ml ($P < 0.05$)

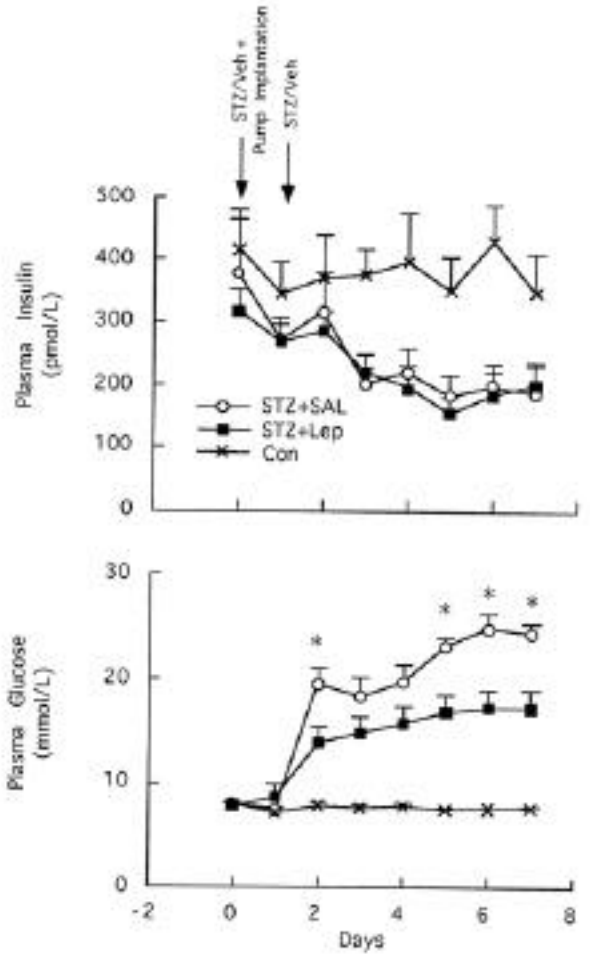

FIG. 1. Plasma insulin and glucose levels in control and STZ-induced diabetic rats. After a basal period (day 0), rats received STZ injections (40 mg/kg) on 2 successive days plus subcutaneous delivery of saline (STZ + Sal) or recombinant rat leptin (STZ + Lep) at 100 $\mu g \cdot kg^{-1} \cdot day^{-1}$ via osmotic minipump. The Con group received Veh and subcutaneous saline. *$P < 0.05$ vs. STZ + Lep.

by day 3 and were reduced by 75% at the end of the study (0.6 ± 0.2 ng/ml, day 7). In the diabetic group receiving recombinant rat leptin, plasma leptin levels increased from 2.6 ± 0.4 (day 0) to a peak of 5.9 ± 0.4 ng/ml on day 1 ($P < 0.05$), presumably because of exogenous leptin infusion superimposed on the basal plasma leptin level. For the final 5 days, however, leptin concentrations in the STZ + Lep group (2.8 ± 0.3 ng/ml) were comparable both to pretreatment values and to levels in the nondiabetic control group, in which the plasma leptin concentration (3.1 ± 0.9 ng/ml, days 0–7) did not change significantly over the course of the experiment. From days 3–7, leptin levels were approximately fivefold higher ($P < 0.05$) in both STZ + Lep and Con rats than in the STZ + Sal group.

Daily food intake (Fig. 2) did not differ among the three groups on day 0 (24 ± 2 g), but declined in each group after minipump implantation (days 1 and 2). In the STZ + Sal group, food intake increased gradually to values 43% greater than basal by day 7 (33 ± 3 g/day, $P < 0.05$). In contrast, daily food intake in the STZ + Lep group did not exceed basal values on any day, nor did it differ significantly from control levels by the end of the experiment. Body weight increased significantly in the STZ + Sal group from 247 ± 10 (day 0) to 258 ± 13 g by day 7 ($P < 0.05$), and in the Con group (from 246 ± 5, day 0, to 266 ± 6, day 7, $P < 0.05$), whereas it decreased nonsignificantly in STZ + Lep from 254 ± 7 (day 0) to 248 ± 9 g (day 7).

Experiment 2. Ten days after either STZ or Veh injection, the STZ-treated rats weighed 338 ± 18 g while the controls weighed 396 ± 12 g ($P < 0.05$). As expected, average daily food intake of the STZ-treated rats was significantly elevated (39.8 ± 4.5 g) compared with controls (27.3 ± 3.7 g, $P < 0.05$). As depicted in Figs. 3 and 4, ICV leptin caused dose-dependent reductions of 4- and 24-h food intake in both groups when compared with synthetic cerebrospinal fluid (CSF) injection. However, the magnitude of the leptin effect did not differ significantly between the two groups. The 4-h food intakes in the group receiving the synthetic CSF injection were 6.8 ± 0.9 in controls and 8.5 ± 1.4 g in the STZ-treated group, while the 24-h food intakes were 28.7 ± 2.4 and 38.1 ± 4.1 g, respectively ($P < 0.05$). In both groups, the lowest leptin dose (0.35 µg) reduced 4- and 24-h food intake by 15% ($P < 0.05$, 24-h food intake), while the two larger doses (1.0 and 3.5 µg) reduced food intake by 22 and 35%, respectively ($P < 0.05$, both time points). Two-way analysis of variance revealed no effect of condition (i.e., STZ versus Veh) on the relationship between leptin dose and the reduction in food intake, indicating that sensitivity to the anorexic effect of ICV leptin is not altered in rats with diabetic hyperphagia.

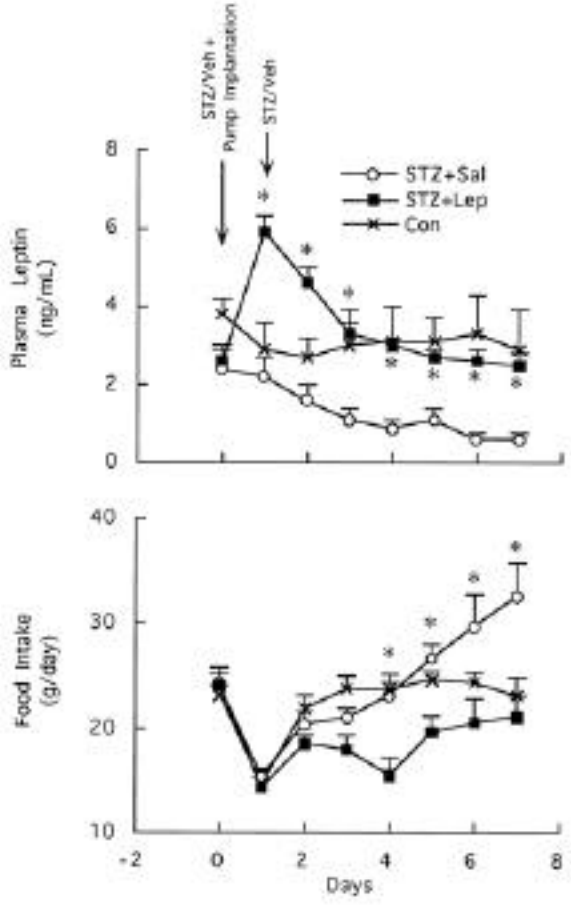

FIG. 2. Plasma leptin levels and daily food intake in control and STZ-induced diabetic rats. After a basal period (day 0), rats received STZ injections (40 mg/kg) on 2 successive days plus subcutaneous delivery of saline (STZ + Sal) or recombinant rat leptin (STZ + Lep) at 100 µg · kg^{-1} · day^{-1} via osmotic minipump. The Con group received Veh and subcutaneous saline. *P < 0.05 vs. STZ + Lep.

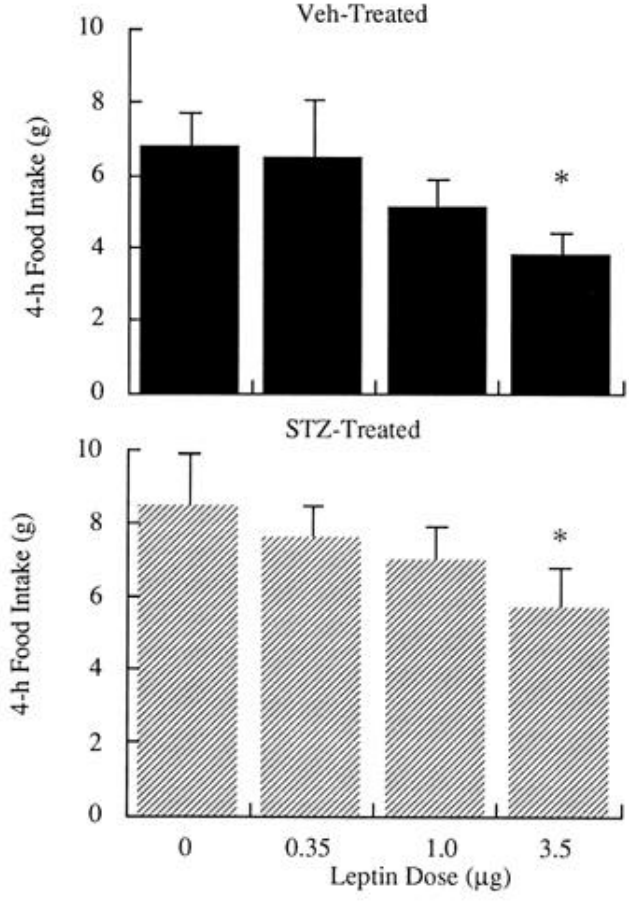

FIG. 3. Food intake measured for 4 h after ICV administration of synthetic CSF or leptin 10 days after injection of STZ or Veh. Each dose of leptin was delivered in 3.5 µl of synthetic CSF 45 min before "lights out," as described in the text. *P < 0.05 vs. 0 µg dose.

DISCUSSION

The pathogenesis of diabetic hyperphagia has been a focus of investigation since it was first documented experimentally more than 30 years ago (29), and many hypotheses have been forwarded to explain the phenomenon (30). Urinary loss of glucose, depletion of body fuel stores, and the inability to use glucose as an energy substrate were each proposed in early studies to explain the stimulatory effect of diabetes on food intake. In the current study, we investigated the role of leptin deficiency in the pathogenesis of diabetic hyperphagia by determining the effect on food intake of systemic leptin infusion at a dose that maintained physiological plasma leptin levels in rats with STZ-induced diabetes. We found that during uncontrolled diabetes in saline-treated rats, marked leptin deficiency developed by day 3, followed by a gradual rise in food intake that peaked at a value 43% above baseline. In contrast, food intake did not increase in diabetic rats in which leptin deficiency was prevented by exogenous leptin infusion. Leptin deficiency, therefore, appears to be required for the development of hyperphagia in rats with uncontrolled diabetes.

In *ob/ob* mice, genetic leptin deficiency is associated with increased hypothalamic expression of the long form of the leptin receptor (31,32) and increased sensitivity to leptin's anorexic effects (33). Hypoleptinemia that occurs in animals with uncontrolled diabetes (23) could therefore increase sensitivity to leptin, which might influence the effect of leptin infusion on food intake. To investigate this possibility, we conducted a dose-response study comparing leptin's ability to suppress food intake after injection into the third ventricle of STZ-treated rats and vehicle-treated nondiabetic controls. Despite the characteristic hyperphagia of STZ-treated rats at baseline, ICV leptin induced a dose-dependent suppression of food intake that was comparable in the two groups (35% suppression with largest dose) and was similar in magnitude to results previously obtained in normal rats (13). These data suggest that STZ-induced diabetes of short duration does not substantially alter sensitivity to leptin-induced anorexia. This finding is in agreement with our recent observation that 7 days of uncontrolled STZ diabetes (P.J.H., D.K.S., M.W.S., unpublished observations) does not increase the hypothalamic expression of leptin receptor mRNA (diabetic group 100 ± 3% vs. control group 100 ± 8%, NS) despite inducing a pronounced decrease of plasma leptin levels. Thus, while it is possibile that longer periods of STZ diabetes would change leptin sensitivity or leptin receptor expression, our results suggest that neither the effect of STZ-induced diabetic hypoleptinemia to increase food intake, nor the effect of physiological leptin replacement to suppress diabetic hyperphagia, can be attributed to a change in sensitivity to leptin's anorexic effects.

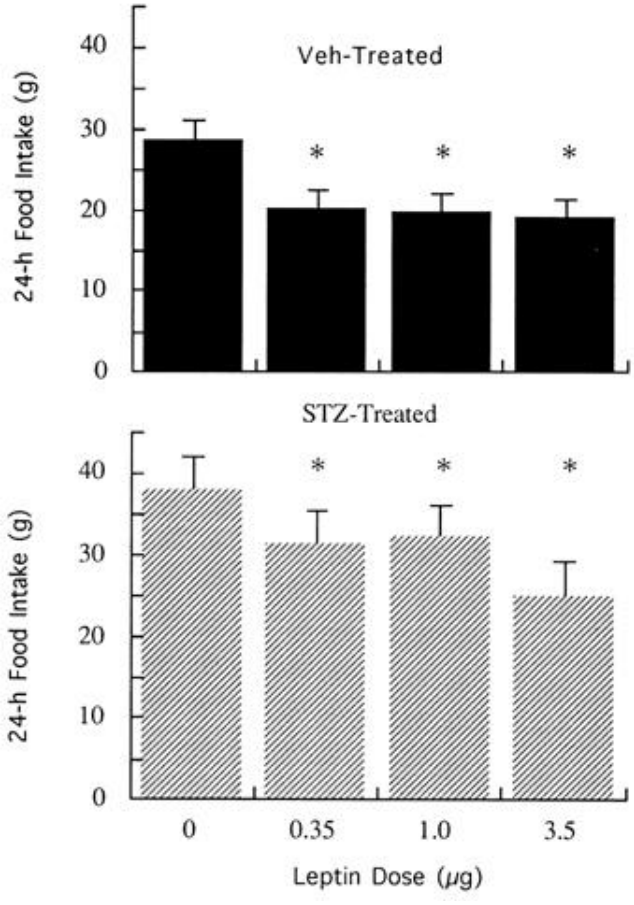

FIG. 4. Food intake measured for 24 h after ICV administration of synthetic CSF or leptin 10 days after injection of STZ or Veh. Each dose of leptin was delivered in 3.5 µl of synthetic CSF 45 min before "lights out," as described in the text. *P < 0.05 vs. 0 µg dose.

Accompanying the prevention of diabetic hyperphagia in the current study was a reduction in the level of hyperglycemia in the STZ + Lep group. One possible explanation for this finding is that the leptin-induced reduction of food intake in diabetic animals resulted in lower plasma glucose levels. However, our data do not exclude the possibility that leptin treatment lowered plasma glucose levels via a mechanism unrelated to reduced food intake. Indeed, the observation that glucose levels were lower in the leptin-treated rats during the first 2 days of leptin infusion, despite similar levels of plasma insulin and food intake in the two STZ diabetic groups (Fig. 2), is consistent with an independent glucose-lowering effect of leptin. Moreover, leptin administration to *ob/ob* mice lowers glucose levels to a greater extent than can be explained by its ability to reduce food intake, apparently via a mechanism that does not require increased insulin secretion (14), and may involve reduced hepatic glycogenolysis (34).

The mechanism by which leptin suppresses food intake in diabetic animals was not addressed in the current study, but may involve changes in hypothalamic neuropeptides previously identified as targets for the action of leptin. For example, hypothalamic expression of mRNA encoding neuropeptide Y (NPY) (35), melanin-concentrating hormone (36,37), agouti-related protein (38), and orexins A and B (39) are each increased in conditions associated with weight loss or leptin deficiency, and each of these peptides exerts stimulatory effects on food intake. Conversely, melanocortins, corticotropin-releasing hormone (35), cocaine- and amphetamine-regulated transcript (40), and thyrotropin-releasing hormone (41) are neuropeptides that inhibit food intake, and each is expressed at reduced levels in rat hypothalamus when leptin and insulin concentrations are reduced (i.e., during fasting). Based on these observations, we hypothesize that peptides that stimulate food intake are overexpressed in the hypothalamus of animals with uncontrolled diabetes, as has been documented for NPY (28), and that this response is attenuated by leptin replacement. Reduced expression of

anorexigenic peptides (as documented for corticotropin-releasing hormone) may also contribute to hyperphagia in diabetic rats. Additional studies are warranted to clarify the hypothalamic response to uncontrolled diabetes and the extent to which it depends on leptin deficiency.

Previous investigations suggest that CNS insulin deficiency plays an important role in the pathogenesis of diabetic hyperphagia (28). This hypothesis is based on the observation that circulating insulin enters the CNS, where it can bind to and activate insulin receptors expressed by neurons in hypothalamic areas implicated in the control of food intake, such as the arcuate nucleus (26,28,42,43). Furthermore, ICV insulin infusion lowers food intake in rats with STZ-diabetes, and this effect is associated with inhibition of hypothalamic synthesis of NPY (28). In the current studies, however, the plasma insulin levels were significantly and comparably reduced in both the STZ + Sal and STZ + Lep groups, suggesting that insulin deficiency, in and of itself, is insufficient to cause hyperphagia in the absence of leptin deficiency. This finding does not exclude the possibility that insulin deficiency contributes to diabetic hyperphagia when leptin levels are low, and further study of the interaction between insulin and leptin in the control of food intake in diabetic animals is warranted.

Many studies have demonstrated that leptin administration lowers food intake in both normal animals and in leptin-deficient *ob/ob* mice (13,14,44,45). Most of these studies, however, investigated the effect of leptin administered at a pharmacological dose or via a nonphysiolgical route (e.g., ICV). The current study is the first to demonstrate that the simple prevention of leptin deficiency is sufficient to prevent the development of hyperphagia in rats with uncontrolled diabetes. This finding provides direct evidence that in genetically normal animals, a decline in leptin from the basal value increases caloric intake, as predicted by the hypothesis that leptin is a major signal to the brain that adipose stores are threatened, as in starvation (35,46).

In summary, we found that STZ-induced diabetes caused a marked decrease of the circulating plasma leptin level that precedes the onset of diabetic hyperphagia and that restoration of normal physiological plasma leptin concentrations prevented the onset of the hyperphagic response. These findings suggest that leptin deficiency mediates, at least in part, the effect of uncontrolled diabetes to increase food intake.

ACKNOWLEDGMENTS

This work was supported by grants NS-32273, DK-52989-01 (M.W.S.), DK-50129, DK-35747 (P.J.H.), DK-54080, DK-54890 (R.J.S.), and DK-17844 (S.C.W.) from the National Institutes of Health/National Institute of Diabetes, Digestive, and Kidney Diseases and the Juvenile Diabetes Foundation International (P.J.H.).

The authors would like to recognize the excellent technical assistance of Hong Nguyen, Kimber Stanhope, Eve Bernstein, and Matt Wonner. The authors would like to thank Amgen, Thousand Oaks, CA, for generously providing the recombinant rat leptin and Hoffmann-LaRoche, Nutley, NJ, for generously providing the human leptin used in the studies.

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