LEPTIN IS A RECENTLY discovered hormone that is synthesized by adipose tissue and regulates, via yet to be defined feedback processes, food intake and fat homeostasis (2, 22). It has been hypothesized that leptin, as a product of adipose tissue, serves as a hormone that indicates the body fat mass and regulates food intake and lipid metabolism. An increase in whole body fat mass is associated with a long-term increase in plasma leptin levels (3, 10, 16), and the resultant hyperleptinemia leads to a reduction in food intake, thus serving as a negative feedback signal to maintain constancy of body fat mass. It has been shown that an acute increase in plasma insulin levels in humans or food ingestion with acute insulin stimulation in rodents increases ob gene expression and/or plasma leptin levels (13, 14, 19).

This observation suggests the existence of a more acute feedback system in which insulin stimulates leptin secretion, which in turn reduces food intake. On the other hand, Caprio et al. (3) were unable to show an acute effect of insulin on leptin secretion. Moreover, Boden et al. (1) required >24 h of hyperinsulinemia to observe a leptin response, whereas Kolaczynski et al. (6) found that 72 h of hyperglycemia-induced hyperinsulinemia was required to observe an increase in plasma leptin levels. Thus conflicting results have been published concerning the effect of insulin on leptin secretion, and little is known about the dose- and time-dependent effects of elevated plasma insulin levels on endogenous leptin secretion. Lastly, although leptin receptors have been demonstrated in the liver (20) and in peripheral tissues, including skeletal muscle and adipocytes (18), it is unknown whether the stimulatory effect of insulin on leptinsecretion is exerted on portal tissues. In the present study we investigated the dose- and time-dependent effect of insulin infusion on peripheral and portal vein plasma leptin concentrations in normal conscious rats. We also examined the effect of prolonged insulin infusion for 7 days on food intake and plasma leptin levels.

MATERIALS AND METHODS

Animals and housing. Male Sprague-Dawley rats of 325–350 g (obtained from Charles River, Wilmington, MA) were subjected to a standard light (0600–1800)-dark (1800–0600) cycle in an air-controlled room (23°C). The rats were housed in individual cages and were given free access to food and water. Four to seven days before the insulin clamp experiments were performed, the rats were anesthetized with pentobarbital sodium (50 mg/kg body wt ip), and indwelling catheters were inserted in the right internal jugular vein and in the left carotid artery; both catheters were exteriorized through the skin at the back of the neck (17). During hyperinsulinemic euglycemic clamps, which lasted for 1 day, rats were permanently connected to a swivel insulin-glucose infusion system (7, 8) and placed in a metabolic cage.

Group I. Hyperinsulinemic euglycemic clamp studies (2–24 h). After an overnight fast, conscious unrestrained rats underwent hyperinsulinemic euglycemic clamps as described previously (7, 8, 17) for 0, 2, 4, 12, or 24 h (5–6 rats/group). During these hyperinsulinemic euglycemic clamps, insulin was administered as a prime (104 µU·kg–1·min–1 over 1 min)-continuous (6 µU·kg–1·min–1) infusion (infusion rate 14 µl/min), and a variable infusion of a 25% glucose solution was started and adjusted to maintain the plasma glucose concentration at ~6 mmol/l, with a coefficient of variation <5% in all studies. Plasma samples for determination of glucose were obtained at 5- to 30-min intervals throughout...
the clamp studies. Plasma samples for insulin and leptin were collected in duplicate at the end of each infusion period. The total amount of blood withdrawn during the clamp study was <5 ml. To prevent intravascular volume depletion and anemia, a solution (1:1 vol/vol) of an equivalent amount of fresh whole blood obtained by heart puncture from littermates of the experimental animal and heparinized saline (10 U/ml) was infused at a constant rate throughout the clamp study. At the end of the study, rats were injected with pentobarbital sodium (60 mg/kg body wt iv), the abdomen was quickly opened, and portal vein blood was taken.

Group II. Hyperinsulinemic (18 mU · kg⁻¹ · min⁻¹) euglycemic clamp studies (2 h). Rats received a 2-h hyperinsulinemic euglycemic clamp using a pharmacological insulin infusion rate of 18 mU · kg⁻¹ · min⁻¹, preceded by a priming insulin dose of 312 mU · kg⁻¹ · min⁻¹ over 1 min. The experimental procedure was otherwise identical to group I rats.

Group III. Hyperinsulinemic euglycemic clamp studies (7 days). Insulin was infused in five rats for 1 wk at a constant rate of 3 mU · kg⁻¹ · min⁻¹, and a 25% glucose infusion was adjusted periodically to maintain euglycemia. Water was available ad libitum, and rat chow was available from 6 PM to 8 AM. Plasma glucose was measured at 9 AM, 12 noon, 3 PM, and 6 PM on a daily basis. Plasma glucose levels were kept close to 7 mmol/l, with a coefficient of variation <10%. During a series of previous experiments, we determined the average glucose infusion rate that was necessary to maintain 24-h euglycemia for 1 wk. Using this as a guideline, we needed only minimal individual adjustments of the exogenous glucose infusion rate to maintain euglycemia during the experiments. The control group (n = 6) received an equal daily volume of vehicle fluids (22 ml/day of 0.3% NaCl) to mimic the water-electrolyte burden in the hyperinsulinemic group.

Every day at 8 AM, 24-h water and food intake was quantified. At 3 PM on days 0, 3, 5, 6, and 7, a 1-ml blood sample was taken for the measurement of plasma glucose, insulin, and leptin concentrations. Blood loss was compensated for by transfusing an equal volume of fresh prewarmed heparinized (50 IU/ml) blood that had been taken from a donor rat by heart puncture. At the end of the study, rats were killed with pentobarbital sodium, and the epididymal fat pads were collected and weighed.

Chemical determinations. Plasma glucose was measured by the glucose oxidase method (Glucose Oxidase Analyzer; Beckman Instruments, Fullerton, CA) and plasma insulin by RIA, using rat and porcine insulin standards (Rat Insulin Kit, Linco, St. Louis, MO). Plasma leptin concentrations were determined by a commercially available RIA, using rat leptin standards (Rat Leptin RIA Kit). The leptin assay was performed with 200 μl of rat plasma, according to the specifications described by Ma et al. (11).

Statistical analyses. ANOVA for repeated measures was used for multiple comparison purposes. When ANOVA showed a significant difference among repeated measurements, Fisher’s least-significant difference test was used for between-group comparisons. Comparisons between just two sets of data were performed with the unpaired Student’s t-test. Associations between plasma leptin levels and food intake in control animals and during the prolonged clamp studies were analyzed with multivariate ANOVA (MANOVA), adjusting for interindividual differences in leptin levels between the animals. The criterion for significance was set at P < 0.05. All data are presented as the means ± SE.

RESULTS

The time-dependent increase in plasma leptin concentration induced by insulin infusion (6 mU · kg⁻¹ · min⁻¹) is shown in Table 1. After 4–12 h of physiological hyperinsulinemia, plasma leptin levels rose significantly in both the peripheral and portal venous circulation and remained elevated after 24 h of sustained hyperinsulinemia (plasma insulin concentration fivefold greater than in overnight fasted rats). During a pharmacological insulin infusion (18 mU · kg⁻¹ · min⁻¹), which raised the plasma insulin concentration to 424 ± 34 mU/l for 2 h, the peripheral and portal vein plasma leptin levels rose to 3.0 ± 0.3 and 3.2 ± 0.6 ng/ml, respectively (P < 0.01 vs. baseline; P not significant, portal vs. peripheral plasma insulin concentration).

Plasma leptin concentrations during prolonged (7 days) insulin infusion (3 mU · kg⁻¹ · min⁻¹) are shown in Fig. 1. At a mean plasma insulin concentration of 107 ± 14 mU/l (∼3-fold over the basal insulin concentration 33 ± 4 mU/l), plasma leptin levels were significantly elevated on days 5, 6, and 7 compared with day 0 and with the control rats (P < 0.05). No significant increase in plasma leptin concentration was observed after 3 days of euglycemic hyperinsulinemia. Plasma glucose concentrations were similar in hyperinsulinemic and control rats (7.4 ± 1.1 vs. 6.7 ± 0.3 mmol/l). Quantitation of 24-h food intake is presented in Fig. 2. On days 6 and 7, a significant decrease in food consumption was observed in hyperinsulinemic rats compared with day 0 and with the control rats (P < 0.05). Using MANOVA,
we observed a negative relationship (P < 0.05) between circulating plasma leptin levels and food intake in the animals receiving prolonged insulin infusion. A 1 ng/ml increase in plasma leptin concentration was associated with a 2.5 g/day decrease in food intake during the 7-day period of hyperinsulinemia. In control animals we observed a positive relationship between plasma leptin levels and food intake (MANOVA, P < 0.05). At the end of the 7-day insulin or vehicle infusion period, the body weights of hyperinsulinemic and control rats were not significantly different (353 ± 10 and 344 ± 12 g, respectively). The weights of the epididymal fat pads were similar in hyperinsulinemic (4.0 ± 0.3 g) and control (4.1 ± 0.3 g) rats.

**DISCUSSION**

In the present study, we have examined the effects of physiological and pharmacological doses of insulin for times ranging from 2 h to 7 days on plasma leptin levels and food intake. Because hypo- and hyperglycemia are known to affect hormonal secretion, appetite, and food intake (9, 15), we employed the euglycemic insulin clamp technique in awake unstressed rats to examine the effect of hyperinsulinemia while maintaining strict euglycemia. Within the physiological range of plasma insulin levels (insulin infusion rate 6 mU·kg⁻¹·min⁻¹, plasma insulin concentration 114–134 µU/ml), at least 4 h are required to observe a rise in plasma leptin levels. Because in humans plasma insulin levels peak at ~1 h after a glucose load and have largely returned to baseline at 2 h, it is unlikely that acute (<2 h) physiological elevations in the plasma insulin concentration have an important role in the regulation of leptin secretion. In contrast, a pharmacological dose of insulin (18 mU·kg⁻¹·min⁻¹) for 2 h is sufficient to induce an increase in plasma leptin concentration.

Fasting peripheral and portal vein plasma leptin concentrations were similar and increased in a parallel fashion after insulin stimulation. This observation indicates that the stimulatory effect of hyperinsulinemia on the release of leptin from visceral adipose tissue is quantitatively similar to the effect of insulin on peripheral adipose tissue. This conclusion is consistent with previous studies that showed that the serum leptin concentration is closely correlated with total body fat mass but not with visceral fat distribution (10, 16). Our studies do not exclude more subtle differences in the ability of insulin to augment leptin secretion differentially by certain adipose depots, including visceral adipose tissue. Thus insulin infusion in rats has been shown to increase leptin mRNA in a site-specific manner in epididymal and perirenal fat pads but not in subcutaneous fat depots (23).

In the present study, we observed a reduction in daily food intake in rats that were exposed to prolonged physiological hyperinsulinemic euglycemia. The decrease in solid food intake closely approximated the increased caloric intake from intravenous glucose, as measured on a cumulative daily basis over the 7-day infusion protocol. Consequently, weight gain and epididymal fat pad size in the insulin-infused rats were similar to those in the control group after 7 days. The decrease in solid food consumption might reflect a physiological response to increased leptin levels. In this case, the increase in plasma leptin would be interpreted to have a negative regulatory effect on appetite (appetite = oral caloric intake). However, if appetite is the reflection of both oral and infused calories, then the rise in endogenous leptin levels during prolonged hyperinsulinemia does not affect appetite (appetite = total caloric intake). From previous studies (2), it is clear that leptin has the potential to reduce caloric intake, but it is unclear whether a prolonged twofold increase in plasma leptin concentration, as seen in the present study, is sufficient to decrease food intake in the absence of insulin and glucose infusion.

As previously reported (14), food intake in normal rats increases obese gene expression. In the present study, we also have shown that higher food intake in normal rats is accompanied by an increase in plasma leptin concentration. Our observations during prolonged hyperinsulinemia clearly show that the relationship between plasma leptin levels and food intake is reversed. During prolonged hyperinsulinemia, the mean plasma leptin level increased approximately twofold, whereas food intake decreased by 50%. It could be determined that a mean increase in plasma leptin of 1 ng/ml was accompanied by a decrease in food intake of 2.5 g/day.

Our failure to observe an increase in endogenous leptin secretion in response to an acute physiological increase in the plasma insulin secretion suggests that enhanced leptin secretion is more closely related to long-term (days) than to short-term (hours) food consumption. This suggests that factors in addition to leptin also have an important role in a complex system that regulates daily food intake (4, 12, 21). This conclusion is in agreement with a study by Karhunen et al. (5), who showed that the serum leptin concentration does not have a role in the short-term regulation of eating in obese women.

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