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Effect of Enteral versus Parenteral Nutrition on Leptin Gene Expression and Release into the Circulation

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Leptin, a putative satiety hormone in rodents, is acutely regulated by fasting and refeeding. To determine the role of satiety hormones that are secreted by the gastrointestinal tract on leptin regulation, leptin mRNA and serum concentrations were measured after feeding rats similar calories with standard chow or infusion of total parenteral nutrition into the duodenum or intravenously. We have demonstrated that leptin gene expression and hormone secretion into the circulation are stimulated equally in the three experimental paradigms; it is unlikely that satiety factors secreted by the intestinal tract play a significant role in leptin regulation. Furthermore, intravenous infusion of individual components of TPN demonstrated that intravenous glucose infusion was mostly responsible for stimulation of the leptin gene and hormone secretion.

In rodents, leptin is a putative satiety hormone that is secreted by adipocytes (1), and is acutely regulated by fasting and refeeding. Fasting results in decreased expression of the leptin gene and circulating leptin levels (2). When a fasted animal is fed standard chow, leptin mRNA levels increase within 3 hours (3-5); to date, meal induced serum leptin levels have not been measured. Since leptin diminishes food intake in laboratory animals (6-8), it has been proposed that an acute rise in leptin serves as a satiety signal. The mechanism of meal induced stimulation of leptin gene expression has been investigated; clearly, in rodents, insulin plays a significant role (9). However, ingestion of meals results in the rise of several nutrients, trace metals, electrolytes and hormones that may also regulate satiety. Several peptides known to stimulate or inhibit feeding are secreted by the gastrointestinal tract (10). These gut hormones are secreted in response to distention of the stomach or to changes in the intraluminal pH or nutrient composition. Many of these satiety hormones are secreted into the circulation. The present study investigates whether satiety hormones secreted by the gut mediate a decrease in food uptake by stimulating leptin gene expression. To this end, we have compared leptin gene expression and hormone secretion in rats fed standard chow, or infused with total parenteral nutrition (TPN) into the duodenum or directly into the venous circulation. We have demonstrated that leptin gene expression and hormone secretion into the circulation is stimulated equally in the three experimental paradigms and makes it unlikely that satiety factors secreted by the intestinal play a significant role in leptin regulation.

METHODS

Animals and diet. Male Sprague-Dawley rats (160-200 g) were housed individually at 24 C with a 12 h light:12 h dark cycle. Rats were trained over 7-10 days to a meal feeding regimen by supplying 8 grams (or 24 Kcal of metabolizable energy) of standard chow (Purina) from 0800 - 1100 in the early light cycle. 12 grams of chow were supplied from 1600 - 1800 at the end of the light cycle and food was withdrawn during the dark cycle.

For another set of experiments, animals undergoing a surgical procedure were fed a standard chow ad lib. Under brief methoxyflurane anesthesia, intraduodenal and jugular vein catheters were placed as previously described (11). The animals were allowed to recover for 3 days. By the second day, all animals were consuming at least 20 g of chow per day. After the third day, food was withdrawn after 1800 hr. The next morning (0800) animals were infused with either dextrose (40%), 2.7 % amino acids (Travasol, Baxter Healthcare Corporation), 15 % intralipid emulsion (Liposyn III, Abbott Laboratories) or TPN (2.7 % Travasol, 40% dextrose, 1% intralipid emulsion, Sodium 45 mEq/l, potassium 30 mEq/l, chloride 30 mEq/l, calcium 5 mEq/l, magnesium 5 meq/l, phosphate15 mM, acetate 35 mEq/l, zinc 5 mg/l, copper 1.0 mg/l, manganese 0.5 mg/l, chromium 1 microgram/l). The infusion rate (6 ml/hr for three hours) was calculated to approximate the number of ingested calories in the animals that ate 8 g of chow in three hours. The total metabolizable energy for each infusion was approximately 27 Kcal for TPN and the intralipid emulsion and 25 Kcal for the dextrose solution.

Northern blot analysis. Epididymal fat pads were harvested and total RNA extracted as a single step isolation method as described

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by the supplier (TRIzol LS reagent, Life Technologies, Gaithersburg, Md.). Total RNA (15 ug) was applied to a 1 % agarose/2M formaldehyde denaturing gel according to standard procedures (12). After electrophoresis, the RNA was transferred to a nylon membrane (Hybond-N, Amersham) by capillary action and fixed by UV light crosslinking. The cDNA probes were radiolabeled with [^{32}P]-dCTP by random primer extension according to the instruction of the supplier (Boehringer Mannheim). Hybridization was performed in a solution as described by the supplier. After 48 hours of incubation at 42 C, the filter was washed twice for 15 min at room temperature in 2 × SSC (20× = 2 M NaCl, 0.3 M sodium citrate, pH 7.0), 0.1 % SDS, and subsequently 2 times in 1 × SSC, 0.1 % SDS for 30 min. at 55 C. The filter was exposed to a phosphorimager (Phosphorimager SF, Molecular Dynamics) cassette overnight and photons from the appropriate bands were measured.

The rat leptin cDNA probe was amplified from 1 μg of total RNA obtained from rat white adipose tissue by the reverse transcription and polymerase chain reaction using primers designed from the published rat sequence (13) (forward: 5′ CCTATCCACAAAGTCCAGGA 3′; reverse: 5′ ATGTCCTGCAGAGAGCCCTG 3′). Probes for other mRNA were isolated from plasmids containing the cloned cDNAs.

Radioimmunoassay. Leptin and insulin levels in serum were measured with the Rat Leptin and Rat Insulin RIA kits, respectively, as described by the supplier (Linco Research, Inc., St. Charles, Mo.). The limit of sensitivity and linearity for the rat leptin assay is 0.5 ng/ml and 50 ng/ml, respectively; for the insulin assay is 0.1 ng/ml and 10 ng/ml, respectively. The low and high quality control (QC) samples were well within 2 S.D.s.

Adipocyte cell isolation and culture. Isolated adipocytes were pooled from the epididymal fat pads of approximately 5-6 male Sprague-Dawley rats that weighed between160-120 g. The fat pads were minced and incubated with 4 mg/ml of collagenase as described by Rodbell,(14) and Marshall (15). After filtering through a mesh and washing, the cells were pooled, divided into equal aliquots, and incubated in DMEM, 0.5% fetal bovine serum and the indicated agents at 37 C, 5% CO_2 for 4 hours.

RESULTS

The effect of ingesting standard chow on adipocyte leptin mRNA levels and serum leptin concentration was determined. Animals were fasted overnight and trained to ingest 8 grams of chow from 0800 - 1100. Each animal ingested all the chow that was provided. The epididymal fat pad was removed and total RNA was extracted in animals that were fasted (0 hr), and 3, 6 and 24 hours after providing food at 0800. A representative Northern analysis of adipocyte leptin and cyclophilin mRNA is shown in Figure 1 (in many autoradiograms an extra band, like that in lane 1, appears between leptin and cyclophilin. When blots are performed with single probes, it becomes apparent that the extra band hybridizes with the cyclophilin probe and may represent a heteronuclear RNA precurser). Leptin mRNA levels (normalized to the house-keeping gene, cyclophilin) acutely increased by 1.7 fold (n=4, $p < 0.01$) 3 hours after providing food and the leptin mRNA returned toward fasting levels by 6 hours. By 24 hrs, leptin mRNA was less than at 0 hr, since animals at 24 hours consumed less chow in the previous 24 hours (8 g vs 20 g) and had a more prolonged fast (21 hrs vs 12 hrs) compared to animals at 0 hrs. Serum levels of leptin varied in parallel

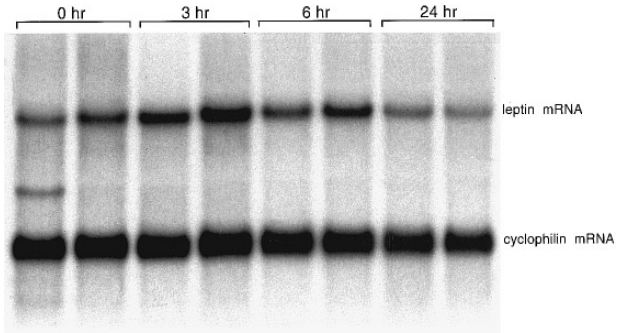

FIG. 1. Effect of feeding on leptin gene expression. Rats were fasted overnight and then trained to ingest 8 g of standard chow from 0800 - 1100 and then food was withdrawn. The animals were sacrificed at 0800 (0 hr), 1100 (3 hr), 1400 (6 hr), and 0800 (24 hr) the next day. Total RNA was extracted and Northern analysis with leptin and cyclophilin cDNA probes was performed as described in the Methods. Each lane was loaded with RNA from one rat.

with adipocyte leptin gene expression. Fasting serum leptin levels were 1.132 ± 0.15 ng/ml. After 3 hrs of feeding, the leptin levels rose to 3.24 ± 0.36 ng/ml (n=4, $p < 0.001$) and then fell to 1.44 ± 0.10 ng/ml 3 hours after eating.

As previously suggested, leptin gene regulatory signals may be derived from distension of the stomach, or absorption of intestinal factors that could be transmitted via gut hormones or the nervous system (3). To determine the contribution of the intestine to meal induced regulation of leptin gene expression, feeding catheters were placed either intravenously (IV) or into the duodenum (ID). Total parenteral nutrition (TPN) was infused and the animals were sacrificed after two hours and the epididymal fat pads extracted. As shown in Figure 2, IV or ID infusion of TPN similarly increased leptin mRNA levels (normalized to cyclophilin) by approximately three fold. Serum leptin levels also increased; fasted animals had serum leptin levels that were undetectable in the assay (less than 500 pg/ml) and rose to 3 ng/ml two hours after the IV or ID infusion of TPN.

The components of TPN were individually infused to determine what nutrient had the greatest impact on leptin gene expression and serum concentration. As shown in Figure 3, intravenous TPN increased leptin mRNA levels by 2.8 ± 0.4 fold ($p < 0.01$). This increase could be almost entirely accounted for by the high concentration of glucose (40%) in the TPN. The leptin gene response to glucose was also dose-responsive. Infusion of 5% glucose at an identical rate increased leptin gene expression by 1.74 ± 0.03 fold ($p < 0.05$). When animals were infused intravenously with 40% glucose at identical rates, leptin mRNA levels rose by 2.6 ± 0.6 ($p < 0.01$). By contrast, the fold stimulation of infused amino acid or lipid components of TPN were minimal (1.1 ± 0.2 or 1.5 ± 0.5, respectively). The serum leptin re-

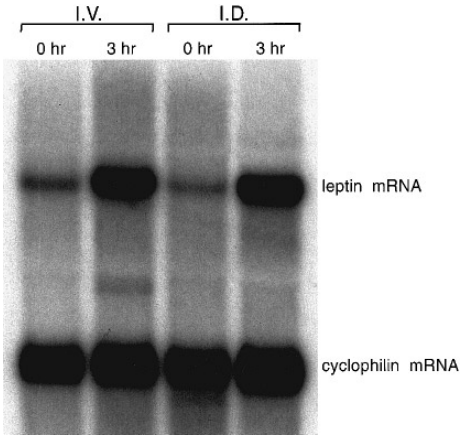

FIG. 2. Effect of TPN infusion intravenously or into the duodenum. Three days before infusion, rats were anesthetized and intravenous and intraduodenal catheters were placed as described in the Methods. The animals were fed *ad lib* until chow was withdrawn the night before the infusion. At 0800 hr, 14 hr fasted rats (o hr) were either sacrificed or infused with TPN intravenously (IV) or into the duodenum (ID) at identical rates (6 ml/hr) for two hours. RNA was extracted and Northern analysis performed as described in the Methods.

sponses to various substrates are shown in Table I and are parallel to the leptin mRNA levels from adipocytes. Serum leptin concentrations rose from undetectable in fasted rats to 2.7 ± 0.02 and 1.9 ± 0.4 ng/ml in TPN and glucose (40%) infused animals, respectively. By contrast, leptin levels rose minimally or none at all in amino acid or intralipid infused animals. Serum insulin levels were also measured in animals infused with various substrates and are shown in Table I. As expected, the greatest rise in serum insulin levels were in animals infused with TPN or glucose. Fat emulsions and the low amino acid concentrations had little or no effect on serum insulin. Since the rise in leptin mRNA and serum concentrations all rise to similar extents in when fed similar calories intravenously or enterally (with either ingestion of chow or intraduodenal infusion of TPN) it appears unlikely that intestinal distention or absorption of intestinal satiety factors has a major role in the acute regulation of the leptin gene. To confirm these observations, we incubated intestinal factors with known satiety properties with isolated rat adipocytes and measured leptin gene expression. As positive controls, adipocytes were incubated for 4 hours with either dexamethasone or insulin; as previously demonstrated, these hormones increased leptin mRNA levels by 200% or 50%, respectively (data not shown). However, no effect could be demonstrated when isolated adipocytes were incubated with 100 ng/ml of calcitonin, cholecystokinin, galanin, enterostatin, neuropeptide Y, peptide YY, pancreatic peptide, vasointestinal peptide, or bombesin.

DISCUSSION

Numerous studies support the theory that the ob gene product, leptin, acts as an acute satiety hormone

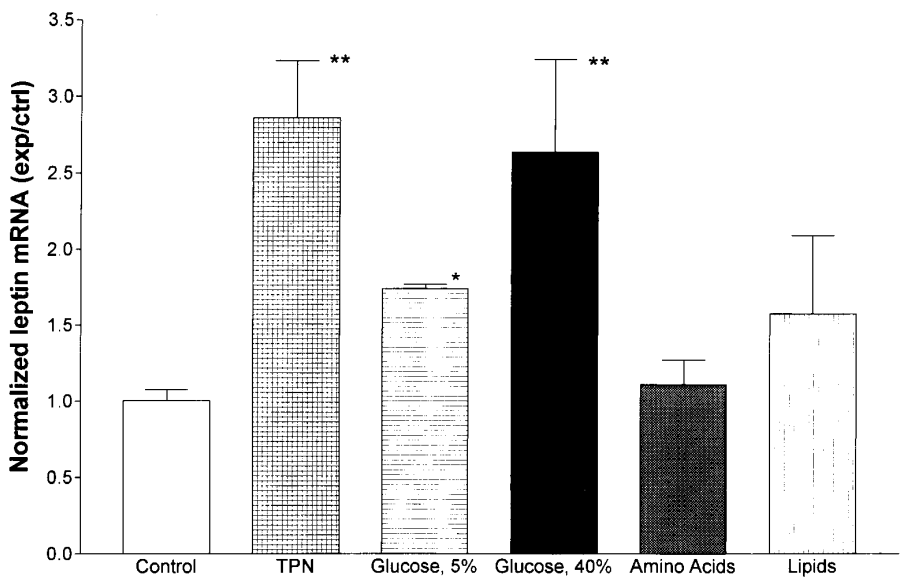

FIG. 3. Effect of intravenous infusion of various substrates on adipocyte leptin gene expression. Animals with central intravenous catheters were fasted overnight and then infused at 0800 with either TPN (n = 4), 5% glucose (n = 3), 40% glucose (n = 3), 2.7 % amino acids (n = 2), or 15 % intralipid (n = 2) for 3 hours at a rate of 6 ml/hour as described in the Methods. Animals were sacrificed before (control) or after the infusions and total RNA extracted from the epididymal fat pads. Northern analysis was performed with leptin and cyclophilin cDNA probes. Normalized leptin mRNA was quantified by dividing the leptin mRNA band by the cyclophilin mRNA band as assessed by phosphorimaging. Each point represents the mean ± S.E.M. of normalized leptin mRNA from infused divided by control animals. * $p < 0.05$ vs Control (Student's t-test). ** $p < 0.01$ vs Control (Student's t-test).

TABLE I

Effect of Infusing Various Substrates on Serum Leptin and Insulin

	Leptin ng/ml (N)	Insulin ng/ml (N)
CTRL	<0.5 (4)	0.5 ± 0.2 (4)
TPN	2.7 ± 0.2 (4)*	6.0 ± 1.6 (4)*
Glucose, 40%	1.9 ± 0.4 (3)*	8.9 ± 1.1 (3)*
Amino acids, 2.7%	1.0 ± 0.05 (2)	0.5 ± 0.1 (2)
Intralipid, 15%	<0.5 (2)	0.8 ± 0.6 (2)

Note. Animals with central intravenous catheters were fasted overnight and then infused at 0800 with either TPN, 40% glucose, 2.7% amino acids, or 15% intralipid for 3 hours at a rate of 6 ml/hour as described in the Methods. Leptin and insulin levels were assayed in serum obtained before (Control) and after the infusion with sensitive radioimmunoassays as described. Each data point represents the mean ± S.E.M.

* $p < 0.01$, compared to control (Student's t test).

in rodents. Animals eat less when infused with intravenous or intraventricular leptin and chronic infusion results in weight loss (6-8). It is also well known that leptin is acutely regulated by fasting and refeeding (2-5). The present study confirms these previous investigations; we have demonstrated that leptin mRNA levels increase approximately two fold when fed 24 Kcal of chow over a three hour period. We have also shown for the first time that feeding results in an acute rise in serum levels, from fasting levels of approximately 1 ng/ml to 3 ng/ml after three hours of feeding. By 6 hours after the beginning of the meal, the serum levels of leptin are close to those observed when fasted.

The mechanism of the acute regulation of leptin gene expression by feeding was further investigated. It is well known that several gastrointestinal peptides act as satiety factors (10). Many of these peptides are secreted by the gut, coordinate digestive functions required for processing a meal and decrease meal size. Probably the best studied peptide is cholecystokinin which reduces meal size in rats when injected either intravenously or intraperitoneally (16,17). Enterostatin also has been shown to reduce food intake in rats (18). Enterostatin is a pentapeptide produced by the cleavage of colipase by pancreatic lipase in the small intestine. Several other gastrointestinal hormones have been demonstrated to affect meal size (for review, see (10)). Before the discovery of leptin, it was thought that these gastrointestinal hormones acted directly on satiety centers located within the central nervous system. However, it was equally plausible that these gastrointestinal peptides may be interacting with a satiety factor, such as leptin, that is secreted from peripheral tissues. Several of our observations have led us to the conclusion that satiety factors released by the gastrointestinal tract have a minimal role in leptin gene expression. Firstly, we observed an equally robust response of the leptin gene and circulating leptin levels when fasted rats were fed TPN enterally or parenterally. Secondly, the leptin gene response and circulating leptin levels were similar in animals fed standard chow or infused intravenously with similar energy equivalents. In both examples, if delivery of nutrients into the duodenum stimulate adipocyte leptin gene expression as their mechanism for satiety, one should have observed a greater response of the leptin gene to enteral vs. parenteral feeding. Finally, *in vitro* incubation of isolated rat adipocytes with various gastrointestinal peptides failed to stimulate leptin gene expression. These studies do not dispute the satiety mediating effect of gastrointestinal peptides. Rather, it appears likely that satiety is determined by multiple mechanisms and that leptin and gastrointestinal peptides have independent roles.

Delivering nutrients intravenously has enabled us to determine what components are important in stimulating leptin gene expression and secretion of leptin into the circulation. Fat emulsions have no effect on leptin gene expression or secretion. This finding contrasts with a mild down-regulation observed when isolated adipocytes were incubated with fatty acids (19). *In vivo* amino acids, delivered in a standard concentration devised to prevent nitrogen loss, have a minimal stimulatory effect on leptin expression and secretion. Larger doses of amino acid infusion were not tested. Most calories delivered via TPN were in the form of glucose; both the infusion of TPN which contains 40% glucose and the infusion of 40% glucose alone stimulated leptin gene expression and circulating leptin levels by greater than two fold. Whether a nutrient stimulated leptin expression or secretion correlated on its ability to stimulate insulin release. This observation is consistent with several other investigations demonstrating the important role of insulin in the acute regulation of leptin gene expression in rodent models. For instance, the fasting-induced decline in leptin gene expression can be reversed with the administration of insulin alone (4). In streptozotocin-diabetic rats, the low levels of leptin mRNA are partially restored with continuous insulin treatment (2). Finally, incubation with insulin of either differentiated 3T3-L1 adipocytes (5,19) or isolated rat adipocytes (20-23). Whether leptin gene expression and hormone secretion are regulated directly by an insulin receptor signaling event or indirectly through insulin's stimulatory effect on nutrient transport and glycolysis remains to be determined.

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