to remain there for 20 s, and were then placed in a holding cage for 30 s until the start of the next trial. After completion of training, the animals returned to their home cages until retention testing 24 h later. On the day of testing, the animals were retained in a waiting room for 1 h before exposure to footshock or systemic injection of corticosterone. Footshock stress was administered in an inhibitory avoidance apparatus (as described elsewhere[9]) in an adjacent, sound-attenuated room. The rats were placed in the starting compartment and, after they stepped completely into the shock compartment, the door between the compartments was closed and a series of three footshocks (0.8 mA for 1 s with a 5-s intershock interval) was administered. After the last footshock, the rats were retained in the shock compartment for 15 s and then returned to their home cages. The animals were assigned randomly to one of the experimental groups. The probe trial consisted of a 60-s free-swim period without a platform and was recorded on video tape for later analysis. The rat was placed in the tank at the arrow (Fig. 1b), a position that was equal in distance to the imaginary target quadrant (T) and opposite quadrant (O). Training and testing was conducted between 10:00 and 15:00 h.

For plasma corticosterone determination, the animals were decapitated immediately after the probe trial and trunk blood was collected in heparinized (500 IU ml^{-1}) tubes and stored on ice. After centrifugation at 5000 r.p.m. for 10 min, the supernatant was stored at −50 °C until assay. Corticosterone plasma concentrations were determined by radioimmunoassay using a highly specific antibody (B3-163, Endocrine Sciences, Tarzana, California) and ^{3}H-corticosterone tracer. Coefficients of variation within and between assays were less than 10%.

Retention performance data were analysed with a two-way ANOVA with treatment as between-subject variable and quadrant as within-subject variable. The ANOVAs were followed by either Fisher's tests for between-subject comparisons or paired *t*-tests for within-subject comparisons. Plasma corticosterone levels were analysed with a one-way ANOVA followed by Fisher's tests. A probability of less than 0.05 was considered significant.

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The stomach is a source of leptin

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The circulating peptide leptin, which is the product of the *ob* gene[1], provides feedback information on the size of fat stores to central Ob receptors[2,3] that control food intake and body-weight homeostasis[4–6]. Leptin has so far been reported to be secreted only by adipocytes[1] and the placenta[7]. Here we show that leptin messenger RNA and leptin protein are present in rat gastric epithelium, and that cells in the glands of the gastric fundic mucosa are immunoreactive for leptin. The physiological function of this previously unsuspected source of leptin is unknown. However, both feeding and administration of CCK-8 (the biologically active carboxy-terminal end of cholecystokinin) result in a rapid and large decrease in both leptin cell immunoreactivity and the leptin content of the fundic epithelium, with a concomitant increase in the concentration of leptin in the plasma. These results indicate that gastric leptin may be involved in early CCK-mediated effects activated by food intake, possibly including satiety.

Oligonucleotides deduced from the cloned mouse *ob* gene[1] were used for screening of total RNA extracted from fundic

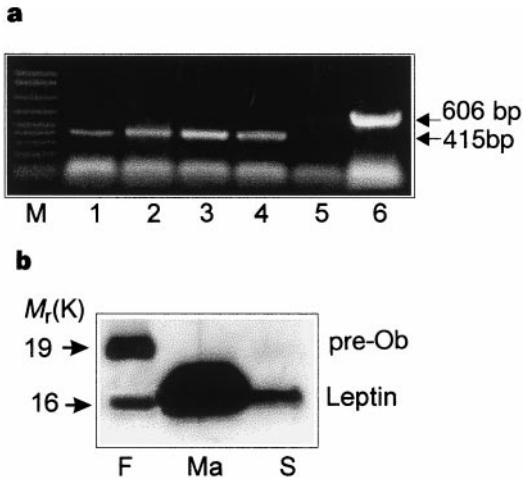

Figure 1 Leptin expression in Wistar rats. **a**, RT-PCR analysis of leptin mRNA: fundic epithelium (lane 1), mesenteric (lane 2), epididymal (lane 3) and perirenal (lane 4) adipose tissues; liver (lane 5); β-actin (lane 6); markers (lane M). Arrows indicate the expected size of the PCR products: 415 bp for *ob* and 606 bp for β-actin. **b**, Western blot of leptin protein: homogenate from fundic epithelium scrapings of Wistar rats (50 μg proteins, lane F), mesenteric adipose tissue (20 μg proteins, lane Ma) and serum (5 μg proteins, lane S). Two leptin immunoreactive bands are seen in the fundic epithelium sample: 16K (leptin) and 19K (leptin precursor, pre-Ob). Note that the 19K band is absent from the adipose tissue and serum.

epithelium scrapings, adipose tissues of various origins (perirenal, epididymal and mesenteric) and liver. Screening was done using the polymerase chain reaction (PCR) after reverse transcription of RNA (RT-PCR). One *ob* PCR product of the expected size of 415 base pairs (bp) was found in fundic epithelium and all adipose tissue tested, but not in liver extract (Fig. 1a). The nucleotide sequence of the amplified product was determined, and was found to be identical to the mRNA product of the *ob* gene[1]. This unexpected finding disagrees with a previous study that failed to detect leptin mRNA in the stomach[1]. However, in this earlier work, total RNA from whole stomach was analysed by northern blotting and RT-PCR, whereas we used RNA that was selectively extracted from the fundic epithelium. Furthermore, we used a much larger amount of total RNA (4 μg compared with 0.1 μg) and more DNA denaturation–renaturation cycles (40 compared with 30 cycles). We did not detect any leptin mRNA in other sites in the gastrointestinal tract, including the small intestine, colon, rectum and pancreas (not shown). The presence of leptin protein in the fundic epithelium was investigated by immunoblot analysis (Fig. 1b). Two immunoreactive bands were observed: a 16K (relative molecular mass, 66,000) band corresponding to leptin and a 19K band, which was probably the leptin precursor[1]. In agreement with previous reports, the 19K protein was absent from extracts of fat tissue. This variance between leptin-containing gastric cells and adipocytes presumably reflects a difference in protein processing and excretion. In addition, the amounts of gastric leptin in lean and obese Zucker rats were similar ($3.8 \pm 0.81\,ng\,g^{-1}$ compared with $4.1 \pm 0.83\,ng\,g^{-1}$ mucosa, respectively), although the latter had more than twice as much epididymal leptin per fad pad (6.3 ± 0.35 ng compared with 22.3 ± 1.5 ng, respectively; $P < 0.001$). This suggests an adipocyte specificity of leptin upregulation in these obese animals, as previously observed for other proteins[8,9].

We found that cells in the gastric epithelium possessed leptin immunoreactivity. They were localized in the lower half of the fundic glands, a site similar to that of the pepsinogen-secreting chief cells (Fig. 2). Leptin immunoreactivity in gastric cells decreased substantially after intraperitoneal administration of CCK-8. In parallel, the leptin content of the fundic epithelium decreased (Fig. 3). The amount of gastric leptin, measured by radioimmune assay (RIA), was slightly decreased by starvation but was not significantly different in rats that had been fasted for 18 h and control animals ($4.3 \pm 0.6\,ng\,g^{-1}$ compared with $5.4 \pm 0.4\,ng\,g^{-1}$, respectively). In contrast, the concentration of leptin in plasma declined sharply during an 18-h fast ($0.52 \pm 0.07\,ng\,ml^{-1}$ compared with $3.12 \pm 0.4\,ng\,ml^{-1}$ in rats fed *ad libitum*; $P < 0.001$). In animals that had fasted for 18 h, CCK-8 produced a dose-dependent decrease in the leptin content of the fundic epithelium. This decrease was significant at a dose of 10 μg kg^{-1} CCK-8 and maximal within 15 min for 30 μg kg^{-1} CCK-8, whereas the plasma leptin level increased rapidly (Fig. 4).

The gastric hormone gastrin, whose structure is closely related to CCK, produced similar effects (not shown). Refeeding of fasted animals also resulted in a rapid and substantial decrease in gastric leptin content (Fig. 4). This decrease reached 66% after 15 min. Thereafter, there was a recovery phase, which probably reflected leptin neosynthesis in the gastric cells. Concomitantly, there was a threefold increase in the concentration of plasma leptin 15 min after the start of refeeding and a fourfold increase after 2 h to a value of 70% of the fed level (Fig. 4). This increase in plasma after two hours of refeeding is consistent with the reported stimulatory effect of refeeding on plasma leptin[10–12] and adipocyte leptin mRNA[13,14]. However, to our knowledge, this is the first report of leptin concentrations being rapidly modulated by both feeding and a peptide.

This finding cannot be explained by adipocyte secretion because adipose cells are not believed to store leptin. Furthermore, in agreement with another report[15], CCK-8 did not stimulate leptin secretion from isolated rat adipocytes, nor did it affect leptin secretion from rat adipocytes transfected with a pCisOb complementary DNA (which directs overproduction of leptin) in comparison to the β_3-receptor agonist BRL37344 which, as previously reported[16,17], inhibited leptin secretion (Table 1). In addition, 15 min after CCK injection, the leptin content of epididymal fat pads showed no significant change (5.42 ± 1.1 ng compared with 6.70 ± 0.8 ng). However, experiments with isolated, vascularized

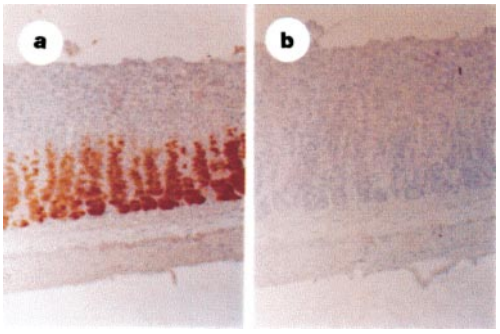

Figure 2 Immunostaining of leptin in rat fundic mucosa. **a**, Leptin immunoreactive cells are seen in the lower half of the fundic epithelial glands. **b**, Serial adjacent section, same region. No immunoreactivity is seen after adsorption of the antiserum with 10 μg ml^{-1} of leptin fragment [137–156].

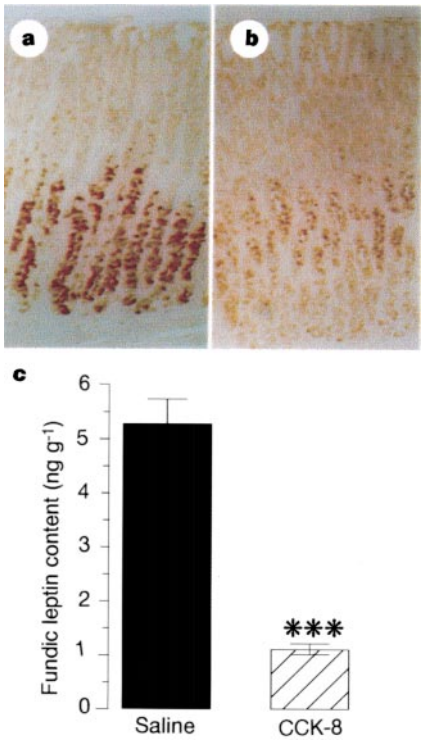

Figure 3 Effects of CCK-8 on immunostaining of leptin in rat fundic mucosa. Fed rats were given an intraperitoneal injection of saline (control) or CCK-8 (300 μg kg^{-1}). In these animals, gastric leptin immunostaining and leptin content were examined 15 min after injection. **a**, A saline-treated rat with cells possessing leptin immunoreactivity seen within the glands, in the lower half of mucosa. **b**, A decrease in immunostaining is evident 15 min after CCK-8 administration. **c**, Fundic content of leptin levels in saline- and the CCK8-treated rats 15 min after injection. Leptin was extracted from the fundic epithelium scrapings and assayed by RIA as described in Methods. Each column is the mean ± 1 s.e.m. for three rats in the control group and five rats in the CCK-treated group. Data were analysed by a Student *t*-test. ***, $P < 0.001$ compared with saline.

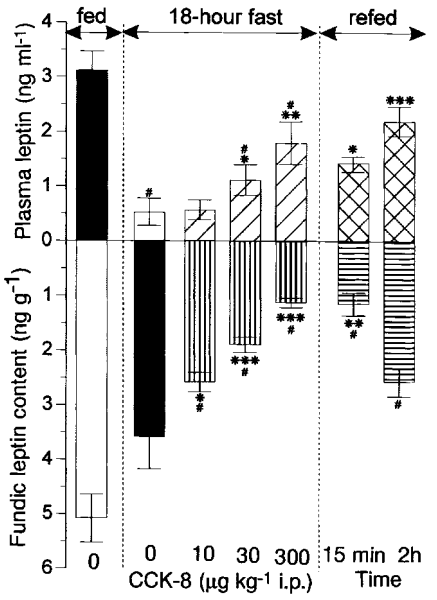

Figure 4 Effects of CCK-8 or feeding on plasma leptin and on fundic content. Wistar rats that were deprived of food for 18 h were injected intraperiotoneally with saline and allowed to re-feed or with CCK-8 (Sigma). Blood was collected from the abdominal aorta at 15-min post-injection after anaesthesia. The stomach was removed and leptin extracted from fundic epithelium scrapings as described in Methods. Each column is the mean ± 1 s.e.m. for six to eight rats. Data were analysed by a Tukey–Kramer multiple comparisons test after a significant ANOVA. #, $P < 0.01$ compared with fed; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control (0).

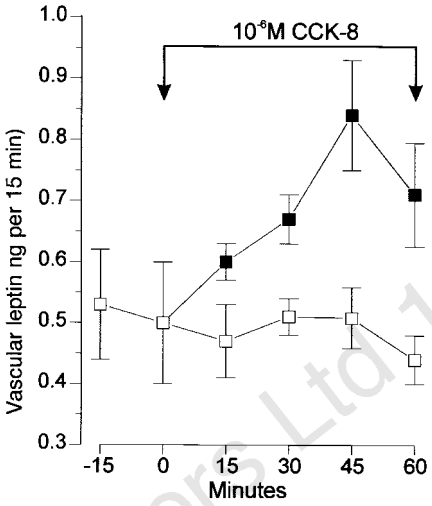

Figure 5 Kinetics of vascular leptin release from the isolated perfused rat stomach. Leptin was assayed in four experiments with CCK-8 (1 μM; filled squares). Basal leptin secretion was measured in three separate experiments (open squares). In the control stomachs, no significant change in leptin concentration was observed in the vascular effluent. Infusion of CCK-8 produced a time-dependent and substantial increase of leptin concentration. Data are means ± 1 s.e.m. and are expressed in nanograms of leptin per 15 min.

stomach (Fig. 5) showed that CCK-induced depletion of gastric leptin content does result in leptin secretion into the blood compartment.

These observations indicate that the rapid increase in the concentration of plasma leptin seen in response to CCK involves the mobilization of a gastric leptin store. RIA determinations with epithelial scrapings (5.4 ng leptin g^{-1}) indicate that this store was about 1.4 ng. However, this value is likely to be an underestimate because of incomplete scraping of the epithelium and proteolytic degradation during scraping. The increase in the plasma concentration was relatively small (0.64 ± 0.09 ng leptin ml^{-1} for 30 μg kg^{-1} CCK-8), and therefore unlikely to have any large, direct effect on satiety control. However, the change could be sufficient to modify the effects of the synergistic interaction with CCK[18,19]. Because of the very rapid degradation of CCK-8 (and CCK) in tissues and blood[20,21], there is a considerable difference between the injected doses and the resulting plasma concentration. Furthermore, CCK released by the intestinal I cells[22,23] in response to feeding probably exerts its satiety effect[24] by local action on vagus afferent neurons[25] and involves local CCK concentrations that may be much higher than the overall concentration in the blood. This is in agreement with the doses of 5–10 μg kg^{-1} that are needed to mimick this effect by intraperitoneal administration of CCK-8[24,25]. These doses are consistent with those that have a significant effect on the release of gastric leptin, but are near threshold values with respect to CCK-induced elevation of plasma leptin, at least under our conditions. Thus, despite being an attractive hypothesis, a role for gastric leptin in CCK-induced satiety remains unproven. Alternatively, gastric leptin may have a local rather than systemic action. Leptin-sensitive vagal afferent terminals have been identified in rat stomach[26]. On the other hand, leptin could enter the liver in quantities sufficiently large to affect glucose production and uptake as well as the hepatic expression of genes encoding key metabolic enzymes[27].

In summary, our findings reveal that the stomach is a source of leptin. Furthermore, the rapid mobilization of gastric leptin by feeding and exogenous CCK-8 indicates that it could be involved in CCK-mediated regulation of gastrointestinal function. □

Table 1 Leptin secretion by isolated adipocytes

	Freshly isolated adipocytes	Transiently transfected adipocytes
	(Secreted leptin, ng ml^{-1} per 2 h)	
Control	0.12 ± 0.01	0.63 ± 0.04
CCK-8 (0.1 μM)	0.13 ± 0.01	0.67 ± 0.02
BRL 37344 (0.01 μM)	ND	0.25 ± 0.02*

Freshly isolated adipocytes or adipocytes overexpressing the leptin gene were incubated for 2 h as described in the Methods. Leptin secretion was measured by RIA. Values are means ± s.e.m. of four determinations in a typical experiment, representative of three experiments. ND, not determined.
*$P < 0.01$ compared with control.

Methods

Animals, tissues and leptin measurements. We used male Wistar, lean (*Fa/fa*) and obese (*fa/fa*) Zucker rats, which were housed in a room at 23 °C with free access to food and water. They were killed by cervical dislocation and white adipose tissues of various origins were collected, together with the liver, stomach and intestine. The stomach and the intestine were opened, rinsed with saline, flattened on a glass plate with the mucosa upwards and the epithelium was scraped off and weighed. Fundic mucosa scrapings and whole epididymal fat pad were homogenized, respectively, in a KRBH solution using a glass–Teflon homogenizer. The homogenates were centrifuged at 10,000*g* for 10 min. The resulting supernatant for fundic mucosa and the infranatant for fat pad were used for leptin assays.

In another set of experiments, rats that had fasted for 18 h were injected intraperitoneally either with 0.9% saline and allowed to refeed on preweighed, standard laboratory pellet chows; or with saline (control) (1 ml per rat) or CCK-8 (Sigma Chemicals, St Louis, Missouri) diluted in 0.9% saline containing 0.1% bovine serum albumin. They were killed at appropriate times and blood was collected from the abdominal aorta, centrifuged and plasma was collected. Leptin concentrations from plasma, fundic mucosa and fat pad were estimated using a radioimmunoassay kit for mouse leptin from Linco Research Inc. (St Charles, Missouri).

PCR primers and RT-PCR conditions. Reverse transcription was done using total RNA extracted by Trizol (Gibco BRL) from fundic epithelium scrapings,

white adipose tissue of various origins, and liver. First-strand cDNA was prepared from 4 μg of total RNA using murine reverse transcriptase according to the Pharmacia Biotech procedure. PCR was done using the following primers: f5′-CCTGTGGCTTTGGTCCTATCTG-3′ and r5′-CTGC TCAAAGC-CACCACCTCTG-3′ (for the *ob* gene) and f5′-CGAGAAGATGA CCCAGAT-CATG-3′ and r5′-AGTGATCTCCTTCTGCATCCTG-3′ (for the β-actin gene). After sample denaturation at 94 °C for 3 min, PCR was done for 40 cycles consisting of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min. The amplification was terminated by a 10-min final extension step at 72 °C. In controls, reverse transcriptase was omitted. Ethidium bromide staining and 1% agarose gel electrophoresis were used to verify the size of the RT-PCR products, that is, 415 bp for the *ob* gene and 606 bp the β-actin gene.

Antibodies. Polyclonal antibody 1 was raised in rabbit against the C-terminal fragment [137–156] of mouse leptin (Santa Cruz Biotechnology), and polyclonal antibody 2 was raised in rabbit against fragment [138–167] of mouse leptin coupled to KLH (keyhole limpet haemocyanin) using EDC (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride). The two antibodies gave similar results in western blot analysis and in histological studies.

Immunoblots. Unfrozen samples of fundic scrapings and mesenteric adipose tissue were homogenized at 4 °C in a RIPA buffer containing 0.1 mg ml^{-1} PMSF, 100 μM benzamidine and 100 mM Na_3VO_4; they were then solubilized in a boiling Laemmli buffer containing β-mercaptoethanol. Proteins were separated by 12.5% polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets and blotted with polyclonal antibody 1. The immune complexes were revealed by using an enhanced chemiluminescence detection system (Amersham).

Immunohistochemistry. Fed rats were injected intraperitoneally with saline (control) or with CCK-8 (300 μg kg^{-1}) and killed after 15 min. Rat stomachs were removed and fixed in Bouin's solution. Sections (4 μm thick) were incubated with antibody 1 diluted to 1 μg ml^{-1} or with antibody 2 diluted 1:50, then with the corresponding biotinylated secondary antibody diluted 1:200, and finally in the avidin–biotin complex diluted 1:100 (Kit ABC Vectastain; Vector Laboratories). Peroxidase activity was revealed by diaminobenzidine. There was no leptin immunostaining in gastric tissues under the following conditions: (1) omission of the primary antibody; and (2) overnight preincubation of the antibodies with various concentrations of the homologous antigen, that is, 10–40 μg of leptin fragment [137–156] (antibody 1) or 10–100 μg mouse recombinant leptin (antibody 2) per millitre of diluted antiserum.

Freshly isolated adipocytes. Adipocytes were isolated from epididymal fat pads by collagenase digestion[28] and incubated as a 30% adipocyte suspension in 500 μl Krebs–Ringer buffer, containing 30 mM HEPES, 1% BSA (KRBH), in the absence (controls) or presence of the various agents for 2 h. The leptin concentration in the medium was then assayed.

Transfected adipocytes. Isolated adipocytes (400 μl of a 50% adipocyte suspension) were transiently transfected by electroporation as described[28], with pCIS2Ob (2 μg) and pCIS2 (8 μg). Cells were diluted in 1.5 ml of Dulbecco-modified Eagle medium (DMEM) containing 0.1% BSA and 25 mM HEPES. They were incubated for 16–24 h at 37 °C in 5% CO_2/95% air, and then pooled, washed once in DMEM, resuspended (20% adipocrit) in KRBH and incubated as above for 2 h.

Isolated, vascularly perfused rat stomach. Male Wistar rats that had been deprived overnight of food were anaesthetized and laparatomized. The stomachs were isolated and vascularly perfused at 37 °C as described[29]. Vascular effluents were collected every 15 min, stored at −20 °C and lyophilized. Samples were reconstituted in RIA buffer before leptin assay.

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Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis

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During endocytosis, clathrin and the clathrin adaptor protein AP-2 (ref. 1), assisted by a variety of accessory factors, help to generate an invaginated bud at the cell membrane[2,3]. One of these factors is Eps15, a clathrin-coat-associated protein that binds the α-adaptin subunit of AP-2 (refs 4–8). Here we investigate the function of Eps15 by characterizing an important binding partner for its