Vagal stimulation exaggerates the inhibitory ghrelin response to oral fat in humans

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Abstract

Ghrelin, the growth hormone secretagogue receptor ligand, is a key regulator of adiposity and food intake. However, the regulation of ghrelin in response to dietary fat intake remains largely unclear. Furthermore, cephalic elevation of ghrelin may influence fat absorption and postprandial lipaemia. Therefore, the aim of this study was to examine the effect of fat ingestion and vagal stimulation on the regulation of plasma ghrelin.

Vagal stimulation was achieved by modified sham feeding (MSF). Eight healthy subjects (four male/four female) consumed a 50 g fat load on two separate occasions. On one occasion, the fat load was preceded by the MSF of a meal for 1 h. Blood, appetite and breath were analysed for 5 h postprandially.

A 25% (S.E.M. $3\cdot4$) suppression in ghrelin concentration was observed after fat ingestion ($P < 0\cdot001$), without an increase in glucose or insulin. MSF in addition to oral fat enhanced ghrelin suppression further, as well as elevating plasma triacylglycerol ($P < 0\cdot001$) and reducing appetite ($P < 0\cdot001$). The fasting ghrelin concentration was inversely correlated with gastric half-emptying time ($P = 0\cdot036$).

We conclude that ghrelin release may be influenced directly by both vagal stimulation and oral fat ingestion.

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Introduction

Ghrelin has recently been identified as the endogenous ligand for the growth hormone secretagogue receptor (Kojima *et al.* 1999, Tschöp *et al.* 2000). Hunger is often noted as a side-effect when i.v. ghrelin is given to increase growth hormone secretion in human subjects (Arvat *et al.* 2001, Wren *et al.* 2001). Administration of ghrelin to rodents has been shown to increase food intake and reduce fat oxidation (Nakazato *et al.* 2001), leading to adiposity (Tschöp *et al.* 2000). Despite the link between adiposity, food intake and plasma ghrelin concentrations, the mechanisms behind ghrelin regulation remain unclear.

Plasma ghrelin is highly suppressed in the postprandial period (Cummings *et al.* 2001, Tschöp *et al.* 2001*a*). To date, postprandial ghrelin concentrations have only been studied following meals containing a significant amount of carbohydrate. Elevated glucose and insulin concentrations have been suggested as potential regulators of postprandial ghrelin (Toshinai *et al.* 2001, Lucidi *et al.* 2002, McCowen *et al.* 2002, Saad *et al.* 2002, Shiiya *et al.* 2002). A more recent study (Schaller *et al.* 2003) has demonstrated that physiological elevations in plasma glucose or insulin concentrations have little effect on the amount of circulating ghrelin. The correlation between body mass index and plasma ghrelin (Tschöp *et al.* 2001*b*) suggests that plasma ghrelin concentrations may be associated with lipid metabolism. Elevation of plasma non-esterified fatty acid (NEFA) concentrations using Intralipid–heparin infusions has no effect on circulating ghrelin concentrations (Mohlig *et al.* 2002); however, this does not necessarily reflect the situation seen following fat ingestion. For example, gastric emptying of nutrients plays a critical role in postprandial ghrelin suppression (Tschöp *et al.* 2000, Williams *et al.* 2003). The extent to which gastric emptying of dietary fat may affect postprandial ghrelin suppression remains to be assessed.

Ingestion of pure fat has been shown to have very little effect on plasma insulin and glucose concentrations (Evans *et al.* 2001), therefore providing a method for assessing postprandial ghrelin regulation, independently of changes in either insulin or glucose concentrations. Oral fat has been previously used to assess the effect of cephalic–phase hormone responses on postprandial metabolism. Cephalic-phase responses are physiological changes initiated by stimulation of the vagus nerve in response to food cues such as the thought, sight, smell and taste of palatable food (Naim *et al.* 1978). The primary metabolic changes occurring in this preprandial period are gallbladder contraction (Hopman *et al.* 1987), gastrin and gastric acid production (Feldman & Richardson 1986), and stimulation of both the exocrine and endocrine pancreas (Konturek & Konturek 2000). The cephalic phase of insulin secretion is now regarded as important in

determining postprandial glucose tolerance (Calles Escandon & Robbins 1987, Teff & Engelman 1996).

Cephalic-phase elevation in ghrelin has been suggested to trigger food intake, and may in part influence the subsequent processing of food. The vagus nerve has been shown to be intrinsically linked to both ghrelin regulation (Lee *et al.* 2002) and action (Masuda *et al.* 2000). Vagotomy elevates plasma ghrelin significantly (Lee *et al.* 2002), whereas electrical stimulation of the vagus nerve tends to decrease ghrelin in rats (Murakami *et al.* 2002). Habitual purging in patients with bulimia nervosa (Tanaka *et al.* 2002) has provided indirect evidence of a potential link between vagal activity and fasting ghrelin concentrations. However, the effects of vagal stimulation on ghrelin regulation have not been properly assessed in humans.

The aim of the current study was to investigate gastrointestinal modulation of plasma ghrelin by examining the effect of fat ingestion and vagal stimulation on postprandial ghrelin secretion in healthy humans. Vagal stimulation was achieved using the modified sham feeding (MSF) technique, as described previously (Jackson *et al.* 2001, Robertson *et al.* 2001, 2002). The maximal effects are achieved when MSF precedes an oral load, effects of which have been shown to extend well into the postprandial period (Robertson *et al.* 2001, 2002).

Materials and Methods

Subjects

Eight healthy subjects (four male, four female) were studied on two separate occasions. The mean age was 22·5 years (range 21- 25) and the mean body mass index was 22·5 kg/m^2 (range 18·2–26·7). Subjects had a fasting mean plasma glucose concentration of 4·9 mmol/l (range 4·0–5·2) and a mean fasting plasma triacylglycerol (TG) concentration of 1·0 mmol/l (range 0·5–1·7). The study was approved by the Oxfordshire Clinical Research Ethics Committee and all subjects gave informed consent before the study.

Study protocol

To standardize the subjects' nutritional state before the study, all the subjects consumed a low-fat (<10 g fat) evening meal to reduce 'spill-over' effects on plasma TG levels (Fielding *et al.* 1996). All subjects were instructed to fast overnight, and to refrain from alcohol and strenuous exercise. A cannula was inserted into an antecubital vein, and a fasting sample was taken at $t=-60$ min. A standard, pure high-fat liquid test meal (oral fat load) containing 466 kJ and 50 g long-chain TGs (SHS International Ltd, Liverpool, UK) was consumed at $t=0$ min. Each subject participated in both test protocols, which were random-

ized. The protocols were as follows: (A) the oral fat load, or (B) the oral fat load preceded by MSF. Blood sampling was performed every 15 min for 60 min preceding the oral fat load, and at 15–30 min intervals for 300 min postprandially.

MSF

All food used for sham feeding was prepared in a room separated from the clinical area. The MSF involved the subjects masticating and expectorating one-quarter of the total test meal every 15 min for a total of 1 h. The MSF was performed at $t=-60, -45, -30$ and -15 min. Subjects were provided with known volumes of water to rinse out their mouths to aid the removal of the food. The meal chosen consisted of a cheese and tomato pizza and a milk and cream drink (38 g fat, 53 g carbohydrate and 2733 kJ). This test meal has been used successfully in other sham-feeding studies (Jackson *et al.* 2001, Robertson *et al.* 2001, 2002). The MSF meal was weighed before and after the study. The expectorated meal was then homogenized in a Waring blender (Dynamics Corporation of America, New Hartford, CT, USA) and a sample was analysed for TG content. Lipids from the meal were extracted in a solution of 2:1 chloroform:methanol followed by transesterification with acidified methanol. Total fatty acid analysis was performed with a Chrompack 9000 gas chromatograph (Varian UK, Walton-on-Thames, UK) with heptadecanoic acid as the internal standard (Fielding *et al.* 1996).

Blood collection and analysis

Blood samples were collected into heparinized syringes (Sarstedt, Leicester, Leics, UK), for analysis of plasma metabolites and insulin. Plasma was separated by centrifugation at 1700 ***g*** for 15 min. Samples for plasma TG, NEFA and insulin analysis were stored at -20 °C until analysed. Whole blood for pancreatic polypeptide (PP) was divided into plain tubes and after clotting, serum was stored at -80 °C until analysis. Blood samples for ghrelin were collected into potassium EDTA-coated tubes containing 200 kIU aprotinin/ml blood (Trasylol; Bayer plc, Newbury, Berks, UK), centrifuged at 1700 ***g*** for 15 min and plasma stored at -20 °C until analysis.

Plasma glucose and TG concentrations were measured with kits from Instrumentation Laboratory (Warrington, Cheshire, UK) and NEFA concentrations were measured with kits from Alpha Laboratories (Eastleigh, Hants, UK). These metabolites were measured enzymatically with an IL Monarch automated analyser (Instrumentation Laboratory). The metabolites were all batch-analysed and exhibited an intra-assay coefficient of variation (CV) of $<2·5\%$. Insulin was measured with a commercially available kit (Pharmacia & Upjohn, Milton Keynes, Bucks, UK).

Serum PP was analysed using a commercially available RIA (Eurodiagnostica, Boldon, Tyne and Wear, UK). Total ghrelin was analysed using a commercially available RIA (Linco, St Louis, MO, USA). RIA antibodies were raised against the C-terminal region (acylated and des-acylated ghrelin) and showed no detected cross-reactivity with motilin-related peptide. The sensitivity of this assay was 10 pg/ml. All samples for hormone analysis were frozen according to the kit manufacturers' instructions. Hormones were batch analysed and exhibited an intra-assay CV of < 10%.

Measurements of gastric emptying

Gastric emptying parameters were calculated using the $[1-^{13}C]$octanoic acid breath test, which has been validated and described in detail previously (Maes *et al.* 1998, Symonds *et al.* 2000). Briefly, $[1-^{13}C]$octanoic acid was added to the oral fat load, for ingestion at t=0 min. Breath samples were taken every 15 min between t=0 min and t=120 min, then at 30 min intervals between t=120 min and t=180 min. Expelled CO_2 was collected in two evacuated glass tubes (Isochem Ltd, Wokingham, Berks, UK) at each time point and analysed using isotope-ratio mass spectrometry. The endogenous CO_2 production during the study was assumed to be constant (Ghoos *et al.* 1993) at 5 mmol/l CO_2/m^2 body surface area per min (Haycock *et al.* 1978). The change in the $^{13}C/^{12}C$ ratio in CO_2 was determined as the difference above baseline compared with the international standard for ^{13}C abundance (Pee Dee Belemnite). The results are expressed as both percentage ^{13}C recovery/min and cumulatively over 180 min.

Visual analogue score (VAS)

Subjects were asked to fill in 'appetite' questionnaires at hourly intervals during the study protocol, as described previously (Flint *et al.* 2000). The questionnaires use a 10 cm graduated line-scale to assess the subject's mood throughout the study period. The questionnaire is designed to measure an individual's eating behaviour and was used to determine if an individual's attitude toward food could be correlated with a particular physiological response.

Statistics

Data were analysed with the Statistical Package for the Social Sciences (SPSS) software version 10·0 (SPSS UK Ltd, Chertsey, Surrey, UK). Normal distribution was assessed using the Shapiro–Wilkes test. Preprandial and postprandial differences in the metabolite and hormone responses between the two protocols were analysed by repeated-measures ANOVA, using 'time' and 'protocol' as the within-subject factors when normally distributed. Area under the curve (AUC) and integrated area under the curve (iAUC) were calculated by using the trapezoidal method. Summary statistics (peak, height, time to peak and AUC) were compared by using a one-way ANOVA combined with a post-hoc Tukey test. Partial correlations (controlling for subject) were also performed between metabolites using Pearson's correlation with a two-tailed significance test. $P < 0·05$ was considered statistically significant. Data are expressed as mean ± S.E.M.

Results

Plasma hormones

Plasma ghrelin concentrations changed in response to both the MSF with oral fat and oral fat alone protocols. Prior to the test meal, there was a rise in plasma ghrelin concentrations. A significantly higher preprandial increase ($P = 0·024$, 143 vs 244 ng/ml change from t= − 60 to t=0, Fig. 1a) was observed with additional MSF (vagal stimulation). There was significant suppression of plasma ghrelin concentration (time, $P < 0·001$) following food intake. In the postprandial period, plasma ghrelin concentrations fell by 517·8 ± 95·0 pg/ml after the fat load alone, whereas there was significantly more suppression (715·0 ± 139·1 pg/ml) with additional MSF (protocol × time interaction, $P < 0·009$). Following oral fat alone, the decline in ghrelin concentration was not immediate, i.e. levels continuing to rise for a further 15 min post-ingestion. This is in contrast to oral fat with MSF, which showed no delay in ghrelin suppression.

Significant PP responses were observed both prepran-dially (protocol, $P = 0·002$) and postprandially (protocol × time, $P = 0·048$, Fig. 1b). A biphasic pattern in PP concentrations was observed during the MSF protocol, with peaks at t= − 30 min (preprandial) and t=15 min (postprandial). The preprandial peak was not observed in the normal fat feeding protocol, but a small postprandial peak was still observed at t=15 min.

Plasma insulin levels remained within the fasting range during both the preprandial and postprandial periods, with no significant difference between the two protocols (Fig. 1c).

Gastric emptying

The effects of MSF and oral fat ingestion on mean rate of recovery of $^{13}CO_2$ in breath after ingestion of labelled octanoic acid are shown in Fig. 2a. The initial rate of recovery was identical for both protocols. By t=75 min, the rate of label recovery was significantly higher after MSF than that of oral fat ingestion alone (protocol × time interaction, $P = 0·001$). When expressed as cumulative recovery (Fig. 2b), the total amount of label recovered after 4 h was higher after MSF compared with fat ingestion
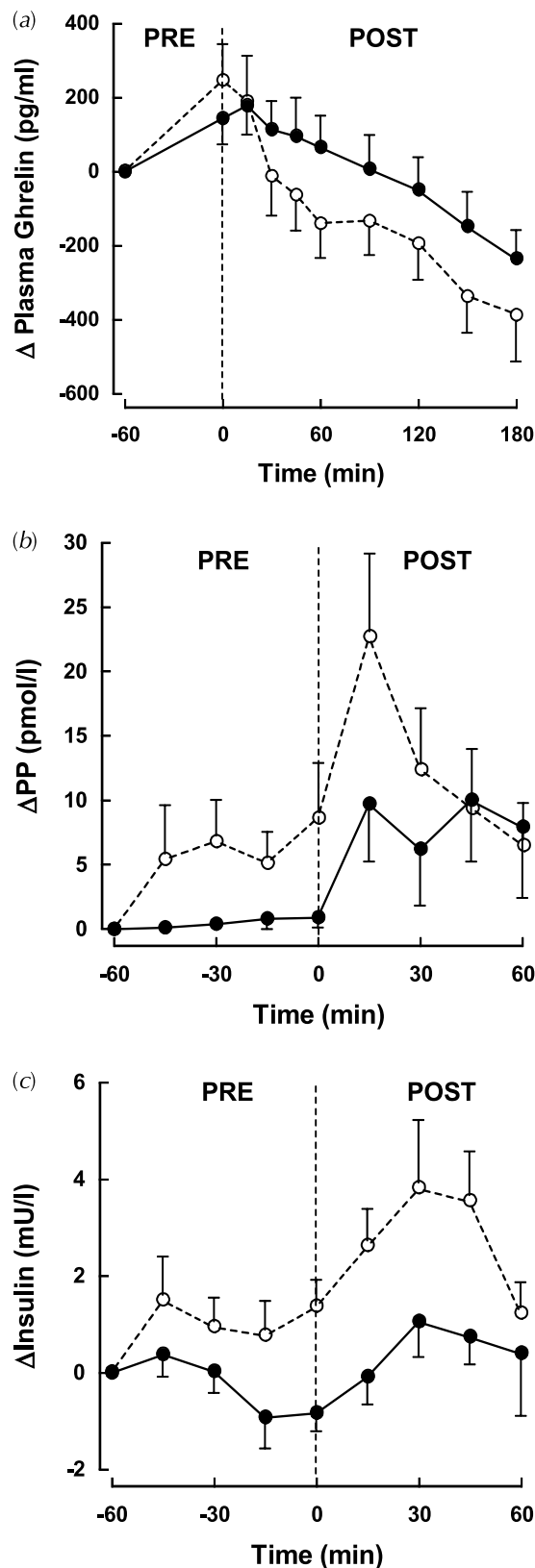
alone (24·1 vs 31·4%). The gastric half-emptying time calculated from the $^{13}CO_2$ recovery data (Ghoos *et al.* 1993) was found to be negatively correlated with the fasting ghrelin concentrations ($r= -0.549$, $P=0.034$).

Plasma TG, NEFA and glucose

In both protocols, there was a significant rise in plasma TG following the oral fat load (time, $P=0.001$), although there was a significantly higher and more rapid peak attained during the MSF with oral fat study ($P=0.001$, Fig. 3a). However, the total TG response as measure by the AUC, was not statistically different between protocols. Peak TG concentration was negatively correlated with the nadir of ghrelin ($P=0.048$, $r=0.523$), whereas fasting TG and ghrelin concentrations showed no correlation ($P=0.382$).

Mean plasma NEFA concentrations were not suppressed after the test meal in either study, but were elevated above baseline in the later postprandial period (Fig. 3B). A significantly earlier postprandial NEFA peak was observed when MSF was performed prior to fat ingestion (protocol × time interaction, $P=0.04$).

Plasma glucose levels remained unchanged in the preprandial period (Fig. 3C). Following fat ingestion, there was a slight dip in glucose levels evident during both protocols.

Expectorated meals

The weights of the MSF meal before and after chewing were compared; the mean (± S.E.M.) recovery was $102.7 \pm 5.2\%$. The recovery of fat content was $96.1 \pm 0.9\%$ as verified by gas chromatography analysis of expectorant and test meal.

Appetite scores

When the subjects were asked 'how satisfied do you feel?' and 'how full do you feel?', they answered that they were significantly more satisfied and more full during the MSF than the control meal (protocol × time $P<0.001$, Fig. 4). When asked other questions, such as 'how much do you

Figure 1 Changes in plasma ghrelin (*a*), PP (*b*), and plasma insulin (*c*) concentrations during an oral fat load preceded by either MSF (○) or no MSF (●). Means ± S.E.M. ($n=8$). The prefix PRE represents preprandial, whereas the prefix POST represents postprandial. For ghrelin concentrations, repeated-measures ANOVA showed a significant effect of prior MSF ($P<0.001$). For PP, repeated-measures ANOVA showed a significant protocol × time effect of prior MSF ($P<0.01$). There was a significant preprandial protocol effect ($P=0.002$) and a significant postprandial (protocol × time effect, $P=0.048$). For insulin, repeated-measures ANOVA showed no significant effect of prior MSF, yet there was a significant time effect ($P<0.001$).
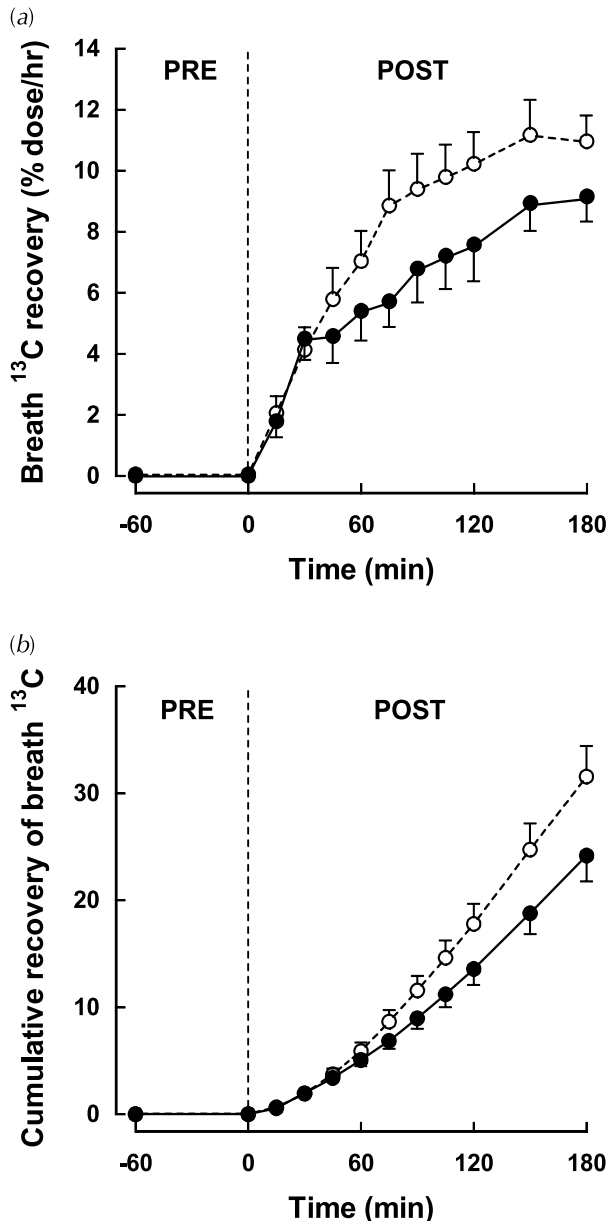
Figure 2 Rate of recovery of $^{13}CO_2$ in breath (*a*) and cumulative recovery of ^{13}C (*b*) after the ingestion of 100 mg [1-^{13}C]octanoic acid in test meal with (○) or without (●) prior sham feeding. Means ± S.E.M. (*n*=8). The prefix PRE represents preprandial, whereas the prefix POST represents postprandial. Repeated-measures ANOVA showed a significant effect of prior MSF (*P*=0·001) on the mean $^{13}CO_2$ recovery rate.

think you can eat?' and 'how hungry do you feel?', there was no statistical difference between the response given on each protocol. The nadir of ghrelin was positively correlated with the sensation of hunger (nadir vs 'how much do you think you can eat' iAUC *r*=0·516, *P*=0·049).
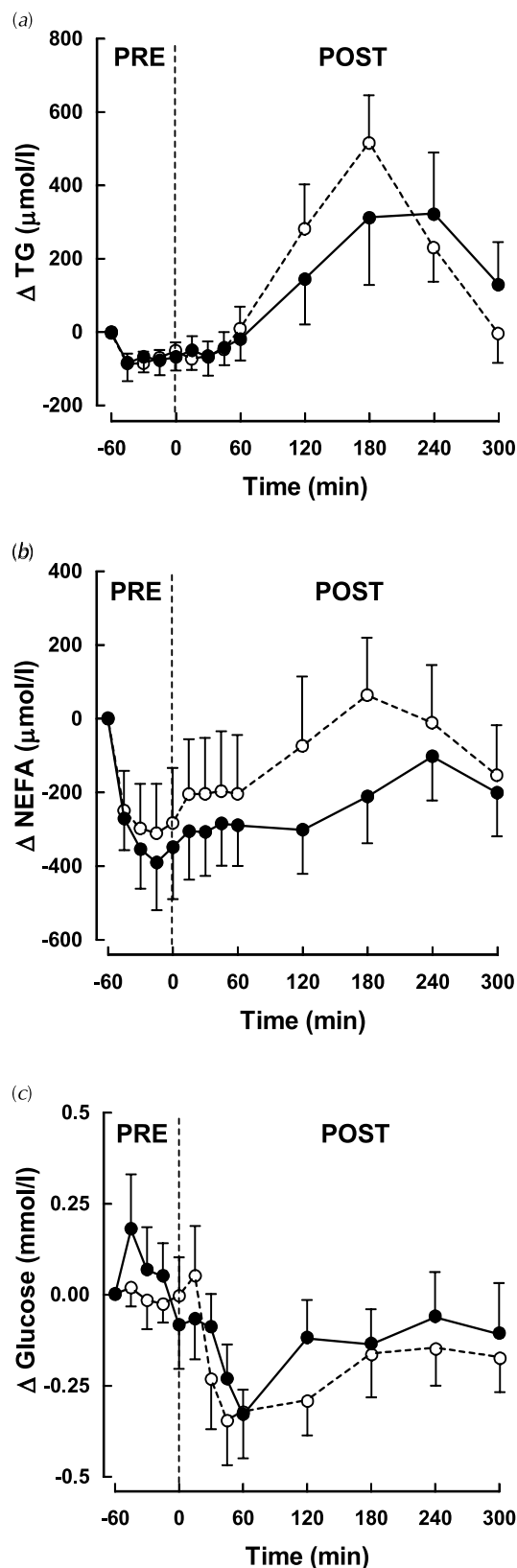
Discussion

A typical preprandial and postprandial pattern in circulating concentrations of ghrelin was observed during each protocol, i.e. a preprandial rise in ghrelin prior to food intake followed by a rapid suppression of ghrelin after oral fat consumption. These effects were observed without alterations in either insulin or glucose concentrations outside the normal fasting range. Furthermore, this pattern of ghrelin secretion was exaggerated following MSF. Exaggerated cephalic-phase elevation of ghrelin was observed, as well as rapid suppression of plasma ghrelin concentrations following oral fat.

The effects of vagal stimulation on fasting ghrelin have been indirectly studied in humans. Habitual purging activity in bulimia nervosa patients is associated with increased afferent vagal activity (Faris *et al.* 2000). Furthermore, patients with bulimia nervosa who are habitual purgers have elevated ghrelin levels compared with those who are not (Tanaka *et al.* 2002). Rodent studies examining the relationship between vagal activity and fasting ghrelin secretion have provided conflicting data. Electrical stimulation of the vagus nerve in fasting rodents caused an increase in circulating concentrations of ghrelin (Murakami *et al.* 2002), whereas a separate study showed an elevation in ghrelin concentrations following vagotomy (Lee *et al.* 2002). However, it must be noted that the measurements of ghrelin were made 14 days after vagotomy, representing long-term changes, rather than the acute changes currently observed. Preprandial elevation of ghrelin is thought to trigger food intake (Cummings *et al.* 2001). Subjects receiving i.v. ghrelin noted an increase in hunger sensations (Arvat *et al.* 2001, Wren *et al.* 2001) and an increase in food consumption when allowed to feed freely (Wren *et al.* 2001). Ghrelin is known to access the hypothalamic arcuate nucleus through a leaky blood–brain barrier, and increases food intake by activating the neuropeptide Y/Agouti–related peptide pathway (Shintani *et al.* 2001, Hewson *et al.* 2002, Lu *et al.* 2002, Traebert *et al.* 2002, Bagnasco *et al.* 2003, Kohno *et al.* 2003, Riediger *et al.* 2003, Seoane *et al.* 2003). Cephalic elevation of ghrelin may upregulate this pathway, leading to not only increased food intake, but also prompt earlier digestive changes such as increased gastrin and gastric acid production (Feldman & Richardson 1986), and stimulation of both the exocrine and endocrine pancreas (Konturek & Konturek 2000), thereby aiding faster processing and absorption of food.

We have demonstrated for the first time that plasma ghrelin levels are significantly suppressed following oral TG. Body mass index has been negatively correlated with fasting ghrelin in numerous studies (Ravussin *et al.* 2001, Tschöp *et al.* 2001b, Ikezaki *et al.* 2002, Shiiya *et al.* 2002), and in the current study (*r*= − 0·836, *P*=0·01). Indeed, fluctuations in weight have been shown to dramatically influence ghrelin concentrations; weight gain decreases

Figure 3 Changes in plasma TG (*a*), NEFA (*b*) and glucose (*c*) concentrations during an oral fat load preceded by either MSF (○) or no MSF (●). Means ± S.E.M. (*n*=8). The prefix PRE represents preprandial, whereas the prefix POST represents postprandial. For TG, repeated-measures ANOVA showed a significant effect of prior MSF ($P<0.001$). For NEFA, repeated-measures ANOVA showed a significant effect of prior MSF ($P=0.04$). For glucose, repeated-measures ANOVA showed no significant effect of prior MSF.

the circulating ghrelin in anorexic patients (Otto *et al.* 2001), whereas weight loss increases 24 h ghrelin concentration profiles (Cummings *et al.* 2002). In rodents, dietary manipulations have shown a decrease in circulating ghrelin following a high-fat diet (Lee *et al.* 2002). In the current study, the suppression of ghrelin appeared to be related to the magnitude of the peak in postprandial plasma TG ($P=0.056$), which could be interpreted as ghrelin regulation being linked to fat absorption. Furthermore, plasma ghrelin was well correlated with the rate of gastric emptying. Oral fat consumption following vagal stimulation led to more rapid gastric emptying, and more rapid suppression of ghrelin than seen with fat ingestion alone. The suppression of ghrelin was associated with increased feeling of fullness and decreased appetite. This highlights ghrelin's role as a regulator of food intake: a preprandial rise in ghrelin release triggers food intake, whereas low postprandial ghrelin levels suppress food intake.

The mechanisms by which the suppression of postprandial ghrelin is regulated are not entirely clear. Following a normal mixed meal, there is an early postprandial elevation of both glucose and insulin concentrations; therefore, it would be likely that ghrelin is suppressed by either of these two signals. However, the effects of glucose and insulin on ghrelin, when assessed using insulinaemic and glycaemic clamps, have been mixed. Insulin and glucose have had no effect on postprandial ghrelin (Caixas *et al.* 2002), whereas insulin suppresses ghrelin independently of glucose concentrations (Lucidi *et al.* 2002, Flanagan *et al.* 2003, Schaller *et al.* 2003). In the current study, the glucose concentrations remained static, whereas the postprandial insulin concentrations remained within the fasting range. This has been previously observed following an oral fat tolerance test (Robertson *et al.* 2002). This suggests that ghrelin can be regulated independently of either insulin or glucose concentrations.

Suppression of ghrelin may be triggered by luminal exposure to free fatty acids in the stomach. Up to 70% of ghrelin production is derived from the stomach (Date *et al.* 2000), although the entire amount of circulating ghrelin can be accounted for by the splanchnic bed (Moller *et al.* 2003). Various gastric secretory and hormonal functions are sensitive to the chemical properties of food within the stomach (McIntosh 1985, Hakanson *et al.* 1986). However, gastric ghrelin-producing cells situated at the base of the mucosal layer are primarily of the closed type, and as
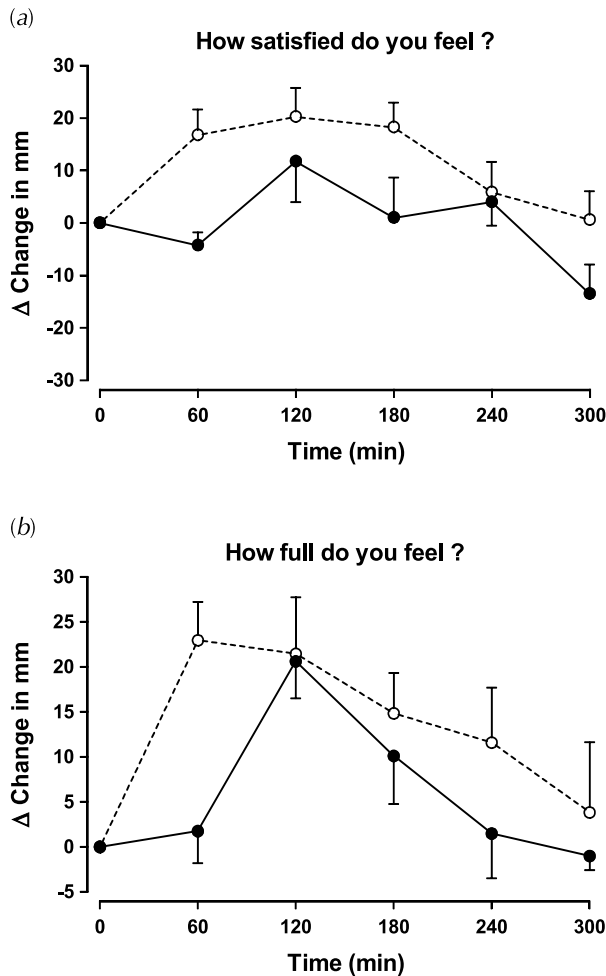
Figure 4 VASs for changes in satisfaction (*a*) and fullness (*b*) during an oral fat load preceded by either MSF (○) or no MSF (●). Means ± S.E.M. (*n*=8). For both satisfaction and fullness, repeated-measures ANOVA showed a significant effect of prior MSF (*P* < 0.001).

The most plausible explanation is that ghrelin is being regulated by the release of another lipid-induced gastro-intestinal hormone. Possible candidate hormones would be small intestinal cholecystokinin (CCK), gastric inhibitory polypeptide and glucagon-like peptide-1 (GLP-1). Ghrelin secretion is influenced by central neural mechanisms originating in the dorsal vagal complex (Lee *et al.* 2002) in the hypothalamus, an area of the brain in which receptors for both CCK (Schick *et al.* 1994) and GLP-1 have been localized (Shughrue *et al.* 1996). However, neither CCK nor GLP-1 is directly affected by cephalic vagal stimulation (Katschinski 2000), yet increased ghrelin suppression occurs following MSF.

In summary, vagal stimulation exaggerates the time-course of ghrelin responses generally seen in response to food intake, i.e. an increase in preprandial ghrelin and rapid postprandial suppression. The physiological consequences are that dietary fat is more rapidly absorbed and metabolized. Cephalic-phase insulin has been suggested as a key regulator of postprandial glucose tolerance. It may be possible the cephalic elevation of ghrelin performs a similar role for postprandial lipid metabolism.

such, are not in direct contact with the gastric lumen (Date *et al.* 2000, Rindi *et al.* 2002, Sakata *et al.* 2002). Furthermore, prevention of gastric emptying of glucose in rodents abolishes the suppression of ghrelin normally seen following oral glucose (Williams *et al.* 2003).

Gastric emptying and the postprandial TG response were closely linked to ghrelin concentrations. This suggests that the entry of dietary fat into the circulation regulates ghrelin secretion. However, in a recent paper, Mohlig *et al.* (2002), using a heparin–Intralipid infusion, failed to affect circulating ghrelin levels, despite a 3- to 4-fold elevation in plasma NEFA concentrations. It cannot be ruled out that ghrelin suppression may be caused by some component of the intestinally derived lipoproteins interacting with the basolateral side of the X/A cell to suppress the release of ghrelin from the small intestine.

References

Arvat E, Maccario M, Di Vito L, Broglio F, Benso A, Gottero C, Papotti M, Muccioli G, Dieguez C, Casanueva FF *et al.* 2001 Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone. *Journal of Clinical Endocrinology and Metabolism* **86** 1169–1174.

Bagnasco M, Tulipano G, Melis MR, Argiolas A, Cocchi D & Muller EE 2003 Endogenous ghrelin is an orexigenic peptide acting in the arcuate nucleus in response to fasting. *Regulatory Peptides* **111** 161–167.

Caixas A, Bashore C, Nash W, Pi Sunyer F & Laferrere B 2002 Insulin, unlike food intake, does not suppress ghrelin in human subjects. *Journal of Clinical Endocrinology and Metabolism* **87** 1902.

Calles Escandon J & Robbins DC 1987 Loss of early phase of insulin release in humans impairs glucose tolerance and blunts thermic effect of glucose. *Diabetes* **36** 1167–1172.

Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE & Weigle DS 2001 A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* **50** 1714–1719.

Cummings DE, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP & Purnell JQ 2002 Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *New England Journal of Medicine* **346** 1623–1630.

Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K & Nakazato M 2000 Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* **141** 4255–4261.

Evans K, Clark ML & Frayn KN 2001 Carbohydrate and fat have different effects on plasma leptin concentrations and adipose tissue leptin production. *Clinical Science* **100** 493–498.

Faris PL, Kim SW, Meller WH, Goodale RL, Oakman SA, Hofbauer RD, Marshall AM, Daughters RS, Banerjee Stevens D, Eckert ED *et al.* 2000 Effect of decreasing afferent vagal activity with ondansetron on symptoms of bulimia nervosa: a randomised, double-blind trial. *Lancet* **355** 792–797.

Feldman M & Richardson CT 1986 Role of thought, sight, smell, and taste of food in the cephalic phase of gastric acid secretion in humans. *Gastroenterology* **90** 428–433.

Fielding BA, Callow J, Owen RM, Samra JS, Matthews DR & Frayn KN 1996 Postprandial lipemia: the origin of an early peak studied by specific dietary fatty acid intake during sequential meals. *American Journal of Clinical Nutrition* **63** 36–41.

Flanagan DE, Evans ML, Monsod TP, Rife F, Heptulla RA, Tamborlane WV & Sherwin RS 2003 The influence of insulin on circulating ghrelin. *American Journal of Physiology. Endocrinology and Metabolism* **284** E313–E316.

Flint A, Raben A, Blundell JE & Astrup A 2000 Reproducibility, power and validity of visual analogue scales in assessment of appetite sensations in single test meal studies. *International Journal of Obesity and Related Metabolic Disorders* **24** 38–48.

Ghoos YF, Maes BD, Geypens BJ, Mys G, Hiele MI, Rutgeerts PJ & Vantrappen G 1993 Measurement of gastric emptying rate of solids by means of a carbon-labeled octanoic acid breath test. *Gastroenterology* **104** 1640–1647.

Hakanson R, Bottcher G, Sundler F & Vallgren S 1986 Activation and hyperplasia of gastrin and enterochromaffin-like cells in the stomach. *Digestion* **35** 23–41.

Haycock GB, Schwartz GJ & Wisotsky DH 1978 Geometric method for measuring body surface area: a height–weight formula validated in infants, children, and adults. *Journal of Pediatrics* **93** 62–66.

Hewson AK, Tung LY, Connell DW, Tookman L & Dickson SL 2002 The rat arcuate nucleus integrates peripheral signals provided by leptin, insulin, and a ghrelin mimetic. *Diabetes* **51** 3412–3419.

Hopman WP, Jansen JB, Rosenbusch G & Lamers CB 1987 Cephalic stimulation of gallbladder contraction in humans: role of cholecystokinin and the cholinergic system. *Digestion* **38** 197–203.

Ikezaki A, Hosoda H, Ito K, Iwama S, Miura N, Matsuoka H, Kondo C, Kojima M, Kangawa K & Sugihara S 2002 Fasting plasma ghrelin levels are negatively correlated with insulin resistance and PAI-1, but not with leptin, in obese children and adolescents. *Diabetes* **51** 3408–3411.

Jackson KG, Robertson MD, Fielding BA, Frayn KN & Williams CM 2001 Second meal effect: modified sham feeding does not provoke the release of stored triacylglycerol from a previous high-fat meal. *British Journal of Nutrition* **85** 149–156.

Katschinski M 2000 Nutritional implications of cephalic phase gastrointestinal responses. *Appetite* **34** 189–196.

Kohno D, Gao HZ, Muroya S, Kikuyama S & Yada T 2003 Ghrelin directly interacts with neuropeptide-Y-containing neurons in the rat arcuate nucleus: Ca^{2+} signaling via protein kinase A and N-type channel-dependent mechanisms and cross-talk with leptin and orexin. *Diabetes* **52** 948–956.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402** 656–660.

Konturek SJ & Konturek JW 2000 Cephalic phase of pancreatic secretion. *Appetite* **34** 197–205.

Lee HM, Wang G, Englander EW, Kojima M & Greeley GH Jr 2002 Ghrelin, a new gastrointestinal endocrine peptide that stimulates insulin secretion: enteric distribution, ontogeny, influence of endocrine, and dietary manipulations. *Endocrinology* **143** 185–190.

Lu S, Guan JL, Wang QP, Uehara K, Yamada S, Goto N, Date Y, Nakazato M, Kojima M, Kangawa K *et al.* 2002 Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. *Neuroscience Letters* **321** 157–160.

Lucidi P, Murdolo G, Di Loreto C, De Cicco A, Parlanti N, Fanelli C, Santeusanio F, Bolli GB & De Feo P 2002 Ghrelin is not necessary for adequate hormonal counterregulation of insulin-induced hypoglycemia. *Diabetes* **51** 2911–2914.

Maes BD, Geypens BJ, Ghoos YF, Hiele MI & Rutgeerts PJ 1998 ^{13}C-octanoic acid breath test for gastric emptying rate of solids. *Gastroenterology* **114** 856–859.

Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M & Kangawa K 2000 Ghrelin stimulates gastric acid secretion and motility in rats. *Biochemical and Biophysical Research Communications* **276** 905–908.

McCowen KC, Maykel JA, Bistrian BR & Ling PR 2002 Circulating ghrelin concentrations are lowered by intravenous glucose or hyperinsulinemic euglycemic conditions in rodents. *Journal of Endocrinology* **175** R7–R11.

McIntosh CH 1985 Gastrointestinal somatostatin: distribution, secretion and physiological significance. *Life Sciences* **37** 2043–2058.

Mohlig M, Spranger J, Otto B, Ristow M, Tschöp M & Pfeiffer AF 2002 Euglycemic hyperinsulinemia, but not lipid infusion, decreases circulating ghrelin levels in humans. *Journal of Endocrinological Investigation* **25** RC36–RC38.

Moller N, Nygren J, Hansen TK, Orskov H, Frystyk J & Nair KS 2003 Splanchnic release of ghrelin in humans. *Journal of Clinical Endocrinology and Metabolism* **88** 850–852.

Murakami N, Hayashida T, Kuroiwa T, Nakahara K, Ida T, Mondal MS, Nakazato M, Kojima M & Kangawa K 2002 Role for central ghrelin in food intake and secretion profile of stomach ghrelin in rats. *Journal of Endocrinology* **174** 283–288.

Naim M, Kare MR & Merrie AM 1978 Effects of oral stimulation on the cephalic phase of pancreatic exocrine in dogs. *Physiology and Behavior* **20** 563–570.

Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K & Matsukura S 2001 A role for ghrelin in the central regulation of feeding. *Nature* **409** 194–198.

Otto B, Cuntz U, Fruehauf E, Wawarta R, Folwaczny C, Riepl RL, Heiman ML, Lehnert P, Fichter M & Tschöp M 2001 Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. *European Journal of Endocrinology* **145** 669–673.

Ravussin E, Tschöp M, Morales S, Bouchard C & Heiman ML 2001 Plasma ghrelin concentration and energy balance: overfeeding and negative energy balance studies in twins. *Journal of Clinical Endocrinology and Metabolism* **86** 4547–4551.

Riediger T, Traebert M, Schmid HA, Scheel C, Lutz TA & Scharrer E 2003 Site-specific effects of ghrelin on the neuronal activity in the hypothalamic arcuate nucleus. *Neuroscience Letters* **341** 151–155.

Rindi G, Necchi V, Savio A, Torsello A, Zoli M, Locatelli V, Raimondo F, Cocchi D & Solcia E 2002 Characterisation of gastric ghrelin cells in man and other mammals: studies in adult and fetal tissues. *Histochemistry and Cell Biology* **117** 511–519.

Robertson MD, Jackson KG, Williams CM, Fielding BA & Frayn KN 2001 Prolonged effects of modified sham feeding on energy substrate mobilization. *American Journal of Clinical Nutrition* **73** 111–117.

Robertson MD, Mason AO & Frayn KN 2002 Timing of vagal stimulation affects postprandial lipid metabolism in humans. *American Journal of Clinical Nutrition* **76** 71–77.

Saad MF, Bernaba B, Hwu CM, Jinagouda S, Fahmi S, Kogosov E & Boyadjian R 2002 Insulin regulates plasma ghrelin concentration. *Journal of Clinical Endocrinology and Metabolism* **87** 3997–4000.

Sakata I, Nakamura K, Yamazaki M, Matsubara M, Hayashi Y, Kangawa K & Sakai T 2002 Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract. *Peptides* **23** 531–536.

Schaller G, Schmidt A, Pleiner J, Woloszczuk W, Wolzt M & Luger A 2003 Plasma ghrelin concentrations are not regulated by glucose or insulin: a double-blind, placebo-controlled crossover clamp study. *Diabetes* **52** 16–20.

Schick RR, Schusdziarra V, Yaksh TL & Go VL 1994 Brain regions where cholecystokinin exerts its effect on satiety. *Annals of the New York Academy of Sciences* **713** 242–254.

Seoane LM, Lopez M, Tovar S, Casanueva FF, Senaris R & Dieguez C 2003 Agouti-related peptide, neuropeptide Y, and somatostatin-producing neurons are targets for ghrelin actions in the rat hypothalamus. *Endocrinology* **144** 544–551.

Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe S, Hosoda H, Kangawa K & Matsukura S 2002 Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. *Journal of Clinical Endocrinology and Metabolism* **87** 240–244.

Shintani M, Ogawa Y, Ebihara K, Aizawa Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M *et al.* 2001 Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes* **50** 227–232.

Shughrue PJ, Lane MV & Merchenthaler I 1996 Glucagon-like peptide-1 receptor (GLP1-R) mrna in the rat hypothalamus. *Endocrinology* **137** 5159–5162.

Symonds EL, Butler RN & Omari TI 2000 Assessment of gastric emptying in the mouse using the [^{13}C]-octanoic acid breath test. *Clinical and Experimental Pharmacology and Physiology* **27** 671–675.

Tanaka M, Naruo T, Muranaga T, Yasuhara D, Shiiya T, Nakazato M, Matsukura S & Nozoe S 2002 Increased fasting plasma ghrelin levels in patients with bulimia nervosa. *European Journal of Endocrinology* **146** R1–R3.

Teff KL & Engelman K 1996 Oral sensory stimulation improves glucose tolerance in humans: effects on insulin, C-peptide, and glucagon. *American Journal of Physiology* **270** R1371–R1379.

Toshinai K, Mondal MS, Nakazato M, Date Y, Murakami N, Kojima M, Kangawa K & Matsukura S 2001 Upregulation of ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. *Biochemical and Biophysical Research Communications* **281** 1220–1225.

Traebert M, Riediger T, Whitebread S, Scharrer E & Schmid HA 2002 Ghrelin acts on leptin-responsive neurones in the rat arcuate nucleus. *Journal of Neuroendocrinology* **14** 580–586.

Tschöp M, Smiley DL & Heiman ML 2000 Ghrelin induces adiposity in rodents. *Nature* **407** 908–913.

Tschöp M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R & Folwaczny C 2001*a* Post-prandial decrease of circulating human ghrelin levels. *Journal of Endocrinological Investigation* **24** RC19–RC21.

Tschöp M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E & Heiman ML 2001*b* Circulating ghrelin levels are decreased in human obesity. *Diabetes* **50** 707–709.

Williams DL, Cummings DE, Grill HJ & Kaplan JM 2003 Meal-related ghrelin suppression requires postgastric feedback. *Endocrinology* **144** 2765–2767.

Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA & Bloom SR 2001 Ghrelin enhances appetite and increases food intake in humans. *Journal of Clinical Endocrinology and Metabolism* **86** 5992.