CLINICAL STUDY

Ghrelin does not orchestrate the metabolic changes seen in fasting but has significant effects on lipid mobilisation and substrate utilisation

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Abstract

Objective: Short-term fasting is associated with increased GH pulsatility and mobilisation of fats, but underlying mechanisms are unclear. We studied ghrelin's role during fasting and the effects of exogenous ghrelin on lipid mobilisation.

Design: Randomised placebo-controlled study.

Methods: In this study, ten controls (body mass index (BMI) 23.3 ± 3.2), ten morbidly obese subjects (BMI 50.1 ± 10.6) and six post-gastrectomy subjects (BMI 25.2 ± 1.0) were fasted for 36 h undergoing regular blood sampling. On a separate occasion, subjects were infused with either i.v. ghrelin (5 pmol/kg per min) or saline over 270 min.

Results: Obese and post-gastrectomy subjects had lower ghrelin compared with controls (ANOVA, $P=0.02$) during the fast. Controls and gastrectomy subjects showed a similar increase in GH pulsatility, circulating non-esterified fatty acids (NEFA) and 3 β -hydroxybutyrate (3 HB). Obese subjects had an impaired GH response ($P<0.001$), reduced excursions of 3 HB ($P=0.01$) but no change in NEFA excursions ($P=0.09$) compared with controls. Ghrelin infusion increased GH, NEFA and ketone bodies (ANOVA, $P<0.0001$) in all the three groups, but GH response was impaired in the obese subjects ($P=0.001$). Ghrelin also induced a significant (ANOVA, $P=0.004$) biphasic NEFA response to meals in all the subjects.

Conclusions: Despite low circulating ghrelin, gastrectomy subjects maintain a normal metabolic response to fasting, implying that ghrelin plays a minimal role. In contrast, infused ghrelin has significant effects on lipid mobilisation and induces a marked biphasic NEFA response to meals. Hence, ghrelin may play a significant role in meal-related substrate utilisation and metabolic flexibility.

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Introduction

It is thought that short starvation periods of 12–24 h were commonplace in ancestral humans and that mammalian endocrine systems partly evolved to maintain energy homeostasis in starvation. The GH/insulin-like growth factor 1 (IGF1) axis plays a key role with augmentation of GH secretion during fasting (1) and GH enhancement of adrenergic-mediated lipolysis and opposition of proteolysis (2). The exact mechanisms underlying these adaptations have not yet been unravelled.

Ghrelin is a 28 amino acid acylated peptide mainly synthesised in the stomach. Ghrelin concentrations are highest during fasting and fall after food ingestion. Furthermore, ghrelin is a powerful stimulant of GH release under experimental conditions (3). However, the significance of ghrelin's role in physiological GH

regulation is unclear. For instance, ghrelin does not appear to mediate the GH response to provocative stimuli such as insulin-induced hypoglycaemia (4). Similarly, the role of ghrelin during short-term fasting has not been clarified. Previous studies have attempted to correlate circulating ghrelin with the GH response, and have come to different conclusions. A clear relationship between GH and ghrelin during fasting was described by two studies (5, 6), whereas further two studies failed to show any correlation (7, 8).

Obese humans are known to have reduced circulating GH levels (9) and an impaired GH response to short-term fasting (10). Obesity is also associated with a reduction in circulating ghrelin, but it is unclear whether this is related to an impaired GH response. Gastrectomy is associated with a marked reduction in circulating ghrelin (11), and although gastrectomy

subjects typically have reduced appetite and difficulty maintaining weight, the GH response to exogenous ghrelin is preserved (12). As a model of markedly reduced circulating ghrelin, it would be of interest to ascertain the metabolic response to fasting in post-gastrectomy subjects.

We, therefore, investigated the metabolic adaptations to short-term fasting in healthy controls, morbidly obese subjects and gastrectomy subjects. If relative ghrelin deficiency leads to an impaired metabolic response to feeding and fasting, then infusion of ghrelin in these subjects may restore it. Hence, we performed a second study in which exogenous ghrelin and saline were infused in a randomised fashion and investigated the metabolic response to feeding in the above three subject groups.

Subjects and methods

Volunteers were recruited by advertisement. The study was approved by the South Sefton Research Ethics Committee (project registration number EC.05.03) and was performed in accordance with the principles of the Declaration of Helsinki. Volunteers gave written informed consent and had a normal physical examination and electrocardiogram. Urea and electrolytes and fasting glucose were normal in all subjects.

Subjects

We studied ten healthy controls aged 25–58 years (mean $\pm$ S.E.M., 42.2 ± 12.0), body mass index (BMI) 23.3 ± 3.2 kg/m², ten obese volunteers aged 31–59 years, BMI 50.1 ± 10.6 kg/m² and six post-gastrectomy volunteers aged 62–71 years, BMI 25.2 ± 1.8 kg/m². Subject numbers were based on recruitment and previously published work examining 24 h fasting ghrelin profiles (13). Obesity was defined as BMI > 30 kg/m² according to the criteria of the World Health Organisation and the International Obesity Task Force. All gastrectomy subjects underwent total gastrectomy for gastric carcinoma at least 12 months previously and were in remission (none had any evidence of recurrence by gastroscopy within the last 12 months). All subjects had a truncal vagotomy as part of the procedure. The mean time between surgery and the study was 5.7 ± 2.6 years (mean $\pm$ S.E.M.). Gastrectomy subjects with significant bile reflux and symptoms of dumping syndrome were excluded. Subjects in all groups with ischaemic heart disease, type 2 diabetes and those aged over 75 years were excluded. Prior to both studies, subjects were asked to refrain from any alcohol or strenuous activity for 24 h.

Protocol

36 h short-term fasting study Subjects ate their usual evening meal at 1900 h and then fasted from 2000 h (day 1). They attended the research unit the

following day at 0830 h (day 2). Blood samples were taken at 0900, 1100, 1300, 1500, 1700, 1900, 2100 and 2400 on day 2 and 0400, 0800, 0830, 0900, 1000, 1100 and 1200 on day 3. Subjects remained fasted, except for water as requested until 0800 h on day 3, when they were served breakfast. Breakfast consisted of 30 g sugared cornflakes, 250 ml semi-skimmed milk, one slice of white bread and a croissant with one carton of margarine and raspberry jam (total energy content 2246 kJ (533 kcal) 67% carbohydrate, 13% protein and 19% fat). These were chosen to mimic a substantial European continental style breakfast, with enough calorific content to elicit a significant hormone response after a prolonged fast. The study was completed at midday on day 3.

Infusion study Subjects were asked to fast and drink only water from 2100 h in the night prior to the study. Each subject was studied on two occasions and received two infusions, 0.9% saline and ghrelin (5 pmol/kg per min), in a randomised, double-blind, crossover design. The dose of 5 pmol/kg per min was chosen, as previous studies have shown infusion of ghrelin at this dose increases food intake in human subjects (14, 15). Human ghrelin was supplied in the acylated (octanoylated) form and produced by Clinalfa Products, Merck Biosciences AG.

The infusion was started at 0830 h ($t=0$) and lasted for 270 min. Previous studies have shown that plasma ghrelin levels during an infusion reach a steady state within 60 min (15). A fixed calorie breakfast was given to all subjects at 60 min following the start of the infusion. This consisted of 40 g cornflakes, 250 ml whole milk, 3.5 g sugar (one sachet) and 100 ml fresh orange juice (1550 kJ, 62% carbohydrate, 13% protein and 25% fat). Subjects consumed the test breakfast within 15 min. They were subsequently offered a free buffet lunch at 1230 (240 min after starting the infusion). The items offered at the buffet lunch were consistent among subjects and were designed to be acceptable to a wide range of palates. Subjects were told that they could eat as much they desired within 30 min. The infusion was stopped at 1300 h after the buffet lunch was completed. Water was freely available during the entire study.

Blood samples were taken at baseline, 30, 60, 75, 90, 105, 120 min and then hourly until the end of the study. The study was completed at 1630. Samples were collected into plastic EDTA tubes containing 0.07 mg aprotinin (500 Kallikrein Inactivator Units (KIU)), centrifuged immediately and then stored at -80°C until assayed.

Assays

For the infusion study, plasma total ghrelin was determined in duplicate by a commercially available RIA (Phoenix Pharmaceuticals, Belmont, CA, USA).

Intra- and inter-assay coefficients were 7.3 and 9.5% respectively. For the fasting study, plasma total ghrelin was measured using an established in-house-specific and sensitive RIA (15–17). The assay could detect changes of 10 pmol/l of plasma ghrelin with 95% confidence limit. The inter- and intra-assay coefficient of variation (CV; at 10 fmol addition) was 6.2 and 9.5% respectively.

Both insulin and GH were measured using a solid phase, two site, chemoluminescent enzyme-labelled immunometric assay run on an Immulite 2000 automated analyser (Diagnostic Products Corporation-UK, Llanberis, Gwynedd, UK, www.dpcweb.com). Glucose was measured by the glucose hexokinase method using the ADVIA 1650 system (Bayer UK Ltd).

Commercial kits were used for measurement of 3 β -hydroxybutyrate (3 HB) ketone bodies and non-esterified fatty acids (NEFA) from Wako (Alpha Laboratories, Eastleigh, Hampshire, UK). For NEFA and 3 HB, the detection limits were 0.003 mmol/l and 3 μ mol/l, respectively, and the inter-assay and intra-assay CV were <10% for both assays.

Statistical analysis

The Statistical Package for the Social Sciences version 14.0 (SPSS, Chicago, IL, USA) was used for data analysis. Mean values were expressed as geometric mean $\pm$ S.E.M. Values that did not follow a normal distribution were log transformed before statistical analysis. Within-group and group interactions with time were analysed using ANOVA for repeated measures. Where violations in parametric assumptions (sphericity) were found within the data set, the within-group and group interactions were measured using a multivariate ANOVA (MANOVA). The Tukey's *b* test was used as a *post hoc* test. Total area under the curve (AUC) was calculated using the trapezoid rule. *P* values <0.05 (two tailed) were considered significant.

Results

Baseline characteristics

Baseline characteristics are summarised in Table 1. The control and obese groups were well matched for age and gender, but the gastrectomy group was significantly older (42.2 ± 12.0 vs 65.8 ± 3.7 , $P < 0.05$). However, no significant correlation between age and fasting ghrelin was found in any of the three groups. The mean BMI in the obese group (50.2 ± 10.6 kg/m²) was significantly higher than that in the control group (23.3 ± 3.2 kg/m²). Weight, waist circumference and body fat percentage were also higher in the obese group. There was no significant difference in BMI, weight, waist circumference and body fat percentage in the gastrectomy group compared with the control group.

Table 1 Baseline characteristics of control, obese and gastrectomy subjects. Values are presented as means $\pm$ S.E.M.

	Control	Obese	Gastrectomy
<i>n</i>	10	10	6
Sex (male %)	50	50	67
Age (years)	42.2 ± 12.0	43.0 ± 8.8	$65.8 \pm 3.7^*$
BMI (kg/m ²)	23.3 ± 3.2	$50.1 \pm 10.6^*$	25.2 ± 1
Weight (kg)	62.7 ± 11.7	$146.0 \pm 45.6^*$	73.5 ± 7.8
Waist (cm)	80.4 ± 9.9	$145.2 \pm 31.4^*$	91.3 ± 11.5
% Body fat	23.9 ± 7.5	$50.2 \pm 10.6^*$	27.9 ± 8.1

* $P < 0.05$ when compared with control values.

Study one

Plasma ghrelin Plasma ghrelin profiles during the 36 h fasting study are shown in Fig. 1a. There was an overall significant difference among the three groups (MANOVA, $P = 0.02$) but *post hoc* tests (Tukey's *b*) comparing group means showed significant differences between control and obese ($P = 0.004$) and control and gastrectomy ($P = 0.002$) but not between obese and gastrectomy ($P = 0.8$). Plasma ghrelin in the lean group significantly changed from baseline over time (ANOVA, $P < 0.001$) as did the obese group (ANOVA, $P = 0.02$), whereas plasma ghrelin did not significantly vary in the gastrectomy group (ANOVA, $P = 0.3$).

Plasma GH GH was released in a pulsatile manner during the fasted and fed state in control and gastrectomy subjects with no significant differences among them (control GH AUC 4322.0 ± 965.2 mIU/l per h versus gastrectomy GH AUC 4155.9 ± 1333.4 mIU/l per h, $P = 0.6$ (Fig. 1b)). Obese subjects, however, displayed a marked reduction in an overall GH release during the fasted and fed state (control GH AUC 4322.0 ± 965.2 mIU/l per h versus obese GH AUC 664.9 ± 111.9 mIU/l per h, $P < 0.0001$).

Plasma NEFA Figure 1c shows the gradual rise in plasma NEFA during the 36 h fast in all the three groups and marked suppression after breakfast on day 3 (ANOVA, $P < 0.001$). The obese group tended to have a lower NEFA rise during the fast, but this did not reach statistical significance (*post hoc* Tukey's *b* test control versus obese, $P = 0.09$).

Plasma 3 HB ketone bodies All the three groups demonstrated a significant rise in 3 HB ketone bodies during the fast (ANOVA, $P < 0.001$) and an overall significant difference among the groups (ANOVA, $P = 0.008$; Fig. 1d). The obese had significantly reduced 3 HB ketone body production during the fast compared with the control (*post hoc* Tukey, $P = 0.04$) and gastrectomy groups ($P = 0.01$), but there was no significant difference between the control and the gastrectomy groups ($P = 0.3$).

Figure 1 Plasma (a) total ghrelin, (b) GH, (c) non-esterified fatty acids (NEFA), (d) 3-hydroxybutyrate (3 HB) ketone bodies, (e) insulin and (f) glucose during the 36 h fast and following re-feeding on day 3 in control, obese and gastrectomy subjects.

Plasma insulin and glucose Plasma insulin tended to be higher in the obese group (Fig. 1e) compared with the control group, but this did not reach statistical significance (control insulin AUC $10\,410.9 \pm 2570.4\,\mu\text{U/ml per h}$ versus obese insulin AUC $18\,182.6 \pm 3029.2\,\mu\text{U/ml per h}$, $P=0.06$). There was no significant difference in insulin response between the control and the gastrectomy groups (control insulin AUC $10\,410.9 \pm 2570.4\,\mu\text{U/ml per h}$ versus gastrectomy insulin AUC $8626.8 \pm 1958.4\,\mu\text{U/ml per h}$, $P=0.6$). Plasma glucose rose after re-feeding with no significant differences among the three groups (Fig. 1f).

Study two

Plasma ghrelin Figure 2a shows plasma ghrelin profiles in the three groups during the saline infusion. There was a significant overall difference in plasma ghrelin among the three groups (ANOVA, $f(2,14)=9.29$

$P=0.03$) and *post hoc* tests (Tukey) showed a significant difference between means of control versus gastrectomy ($P<0.001$), obese versus gastrectomy ($P=0.01$) but not control versus obese ($P=0.08$). Fasting ghrelin values were also significantly different among the three groups and are shown in Table 2. The control group showed a significant nadir in ghrelin release following the fixed energy breakfast and the buffet lunch (significant changes from baseline at 120 min ($P=0.05$) and at 360 min ($P=0.02$) after the start of the infusion), whereas the obese group had no significant suppression in plasma ghrelin after meal ($P=0.2$) but interestingly had a significant rise in ghrelin pre-lunch (significant change from baseline at 240 min after the start of the infusion ($P=0.02$)). The gastrectomy group contrarily did not show any significant change from baseline in plasma ghrelin throughout the infusion study. Plasma ghrelin levels during the ghrelin infusion reached steady state after 60 min (Fig. 2b). There was no significant difference between the mean steady state plasma ghrelin

Figure 2 Plasma ghrelin during (a) saline and (b) ghrelin infusion in control, obese and gastrectomy subjects.

and the AUC in any of the three groups (Table 2). It should be noted that as stated in the methods, plasma ghrelin during the infusion study and the fasting study were measured by two different immunoassays, and hence, there are differences in absolute values.

Plasma GH GH levels were low during the saline infusion but showed a marked GH response during the ghrelin infusion in all the three groups. The obese group had a significantly impaired GH response compared with the control group (Fig. 3A; AUC control $30\,095.8 \pm 4701.8$ mIU/l per min versus obese 7998.2 ± 1308.5 mIU/l per min, $P=0.001$), but there was no difference in GH response between the control and the gastrectomy subjects (AUC control $30\,095.8 \pm 4701.8$ mIU/l per min versus gastrectomy $30\,972.6 \pm 4556.4$ mIU/l per min, $P=0.89$; Fig. 3A).

Plasma NEFA ANOVA for repeated measures showed an overall highly significant effect of the ghrelin infusion on plasma NEFA ($f(1,11)=35$, $P<0.001$). Fig. 4a–c demonstrate the effects of ghrelin on plasma NEFA in the three individual subject groups. In the control group, the difference in AUC between the ghrelin and the saline condition did not reach statistical significance ($P=0.06$), but *post hoc* tests showed significant differences at 120 min ($P=0.04$) and 240 min ($P=0.02$). In the obese subjects, there was a significant difference in AUC between ghrelin and saline ($P=0.01$) and *post hoc* tests showed a significant difference at 240 min ($P=0.04$). Ghrelin appeared to have a marked effect on plasma NEFA in the gastrectomy group with significant difference in AUC ($P<0.01$) between ghrelin and saline and at time points $t=0$ min ($P=0.02$), 60 min ($P=0.01$), 180 min ($P=0.01$) and 240 min ($P=0.02$).

Interestingly, there was a highly significant interaction between time and the ghrelin infusion (ANOVA, $f(6,6)=11.6$, $P=0.004$) suggesting that plasma NEFA only varied significantly from baseline during the ghrelin condition. Indeed, there appeared to be a biphasic response of plasma NEFA to the ghrelin infusion with a small rise in plasma NEFA before each meal. In the control subjects, there was a non-significant rise in plasma NEFA before breakfast ($P=0.17$), but a significant rise in plasma NEFA before lunch ($P=0.04$); obese subjects contrarily showed a far smaller increase in plasma NEFA before each meal and this did not reach statistical significance (increase before breakfast, $P=0.5$; increase before lunch, $P=0.17$). Gastrectomy subjects demonstrated a marked biphasic response with a significant increase before breakfast ($P=0.004$) and before lunch ($P=0.01$) during the ghrelin condition. None of the groups showed a significant increase in plasma NEFA prior to meals in the saline condition.

Plasma 3 HB ketone bodies Figure 4d–f show that the ghrelin infusion had a significant effect on 3 HB ketone bodies (ANOVA, $f(1,9)=6.4$, $P=0.03$). All the three groups showed a significant increase in plasma 3 HB ketone bodies before breakfast during the ghrelin infusion (control, $P=0.02$; obese, $P=0.04$; gastrectomy, $P=0.02$) but not during the saline infusion. In contrast to the effect of ghrelin on plasma NEFA, there was no similar biphasic response with plasma 3 HB ketone bodies and ghrelin had no effect on the temporal profile in any of the three groups (ANOVA, $P=0.28$).

Table 2 Plasma ghrelin during the ghrelin infusion. Values are presented as means $\pm$ S.E.M.

Plasma ghrelin	Control	Obese	Gastrectomy
Fasting (pg/ml)	762 ± 61	$414 \pm 86^*$	$226.1 \pm 62^*$
Steady-state (pg/ml)	5526.8 ± 65	6137.4 ± 469	5559.6 ± 209
AUC (ng/ml per min)	1.5 ± 0.2	1.69 ± 0.2	1.5 ± 0.1

* $P<0.05$ when compared with control values. AUC, area under the curve.

Figure 3 (a) Comparison of the plasma GH responses among the three groups during the ghrelin infusion. Comparing the ghrelin and saline infusions, plasma GH responses are shown in figures (b) control, (c) obese and (d) gastrectomy subjects.

Plasma insulin and glucose Ghrelin infusion had a significant effect on glucose (ANOVA, $P < 0.001$), increasing post-prandial values in all the three groups (Fig. 5a–c). Ghrelin increased serum insulin levels in the healthy controls (AUC insulin during ghrelin $8608 \pm 507 \mu\text{U/ml per min}$ versus AUC insulin during saline $6604 \pm 696 \mu\text{U/ml per min}$ $P = 0.04$) but not significantly in obese and gastrectomy subjects (Fig. 5d–f). To control for differences in glucose, the effect of ghrelin on the insulin/glucose ratio was also calculated. There was no significant effect of ghrelin on insulin/glucose ratio in any of the three groups (AUC insulin/glucose ratio during ghrelin $858 \pm 43 \text{ min}$ versus AUC insulin/glucose ratio during saline $911 \pm 101 \text{ min}$, $P = 0.6$, in healthy controls).

Table 3 shows a summary of the main effects in the three subject groups.

Discussion

Our study is the first to show that gastrectomised subjects maintain a normal physiological response to a short-term fast despite abnormal low circulating ghrelin. We have shown that supraphysiological levels of ghrelin have significant effects on lipid metabolism and demonstrated for the first time that ghrelin induces a biphasic NEFA response to meals. We have also confirmed previous data showing that obese subjects have impaired fasting and ghrelin-induced GH release and that ghrelin-induced GH release is preserved in gastrectomised subjects.

It is interesting to observe that obesity and the gastrectomised state are different in terms of GH secretion during fasting. Despite significantly low circulating ghrelin, gastrectomised subjects displayed a similar GH profile to lean controls during the fast, whereas obese subjects had a markedly impaired GH response to fasting, confirming the findings of a previous study (18). Therefore, it is likely that factors other than ghrelin modulate the GH response to fasting in obesity. Following on from this, ghrelin infusion induced a significant surge in GH release in all the three groups but this was significantly impaired in the obese subjects, as reported by other groups (14). It is unclear why, in the obese state, ghrelin is less effective at stimulating GH. Leptin is a possible candidate, but previous work has shown that leptin-deficient human subjects have similar GH responses to GH releasing peptide 6 (GHRP6) as BMI-matched controls (19). Similarly, a vagally mediated signal is plausible, but it is noteworthy that vagotomised subjects have an intact ghrelin–GH response; presumably, a factor unique to adipose tissue is involved. Like others (12), we have shown that gastrectomised subjects have a preserved GH response to ghrelin; this confirms that the receptors mediating the GH response to ghrelin are outside the stomach and that the response does not require

Figure 4 Plasma non-esterified fatty acids (NEFA) during ghrelin and saline infusions in (a) control, (b) obese and (c) gastrectomy subjects and 3-hydroxybutyrate (3 HB) ketone bodies during the ghrelin and saline infusions in (d) control, (e) obese and (f) gastrectomy subjects. *Significant difference at that particular time point ($P < 0.05$).

an intact vagus; indeed, these receptors may be up-regulated following gastrectomy.

We have also provided further insight into the mobilisation of fatty acids and ketone bodies during fasting. Our study confirmed early studies that showed obese subjects have an attenuated rise in NEFA and ketone bodies during fasting (20, 21). Circulating ghrelin levels were also lower in the obese subjects during the fast, but it seems unlikely that these are linked to impaired GH, NEFA and ketone body production, as gastrectomy subjects with still lower ghrelin levels have an intact starvation response. Obese subjects, therefore, have an impaired response to starvation for reasons, which may involve hyperinsulinaemia and insulin resistance but are yet to be clarified and are not likely to be secondary to relative deficiency in ghrelin.

Rises in plasma NEFA and 3 HB ketone bodies during the fast were preserved in gastrectomised subjects. It is probable that other compensatory central mechanisms unique to post-gastrectomy patients are responsible for the preservation of these important metabolic changes – indeed, previous animal studies have confirmed that sensitivity to cholecystokinin (CCK) alters post-gastric surgery (22). Similarly, Kim *et al.* (23) have shown that hypothalamic ghrelin receptor expression increases

eightfold in fasted rodents. It is plausible that gastrectomised subjects have increased sensitivity to ghrelin, hence maintaining a response to starvation, and that this sensitivity is normal or lower in obese subjects. To explore this, further dose-response infusion studies would be needed, and indeed, this would be an interesting area for future research. Nonetheless, our current data suggests that ghrelin is not the driving force behind the metabolic changes observed in short-term fasting in this group. We also know that 60–70% of gastrectomy subjects are permanently below ideal body weight (24) for unknown reasons, and yet, this data demonstrates that their response to short-term starvation is intact.

Although the fasting study suggests that ghrelin probably has little physiological role in mobilising fatty acids and forming ketone bodies during a fast, our infusion study certainly suggests that ghrelin, at least at the supraphysiological levels achieved during this study, does have a significant effect on fatty acid release and ketone body formation. Ghrelin increased circulating NEFA during the infusion study consistent with other published data (25). During the ghrelin infusion, all the three groups also appeared to show a biphasic NEFA response, with an anticipatory rise and subsequent fall after each meal. This was most pronounced in the

Figure 5 Plasma glucose during the ghrelin and saline infusions in (a) control, (b) obese and (c) gastrectomy subjects and insulin during the ghrelin and saline infusions in (c) control, (d) obese and (e) gastrectomy subjects. *Significant difference at that particular time point ($P < 0.05$).

control and gastrectomised groups. The obese group graphically appeared to show a biphasic response, but this was considerably blunted and the pre-lunch rise was not significant. While we would accept that there were no further samples taken in the hour prior to lunch, the increased variation in NEFA was highly significant despite small numbers. It is also of interest to note that in the fasting study, the control and gastrectomy groups appear to show an anticipatory

rise in NEFA prior to the second meal, which is not present during the saline condition of the infusion study – despite a similar time period between breakfast and lunch. Again, this is slightly blunted in the obese group. Has the prolonged fast increased sensitivity to ghrelin and hence reproduced a similar effect to supra-physiological ghrelin? Certainly, the circulating NEFA levels are higher in the fasting study and, to a lesser extent, this was also the case during the ghrelin infusion.

Table 3 Summary of hormonal responses in the three groups during the fed and fasted states.

	Control	Obese	Gastrectomy
Fasted state			
Ghrelin	Significant change from baseline	Decreased*	Decreased*
GH	Significant change from baseline	Decreased response*	Similar response to control
NEFA	Increase	Similar response to control	Similar response to control
3 HB ketone bodies	Increase	Decreased response*	Similar response to control
Insulin	No change	Similar response to control	Similar response to control
Glucose	No change	Similar response to control	Similar response to control
Effect of ghrelin during fed state			
GH	Increased	Decreased response*	Similar response to control
NEFA	Biphasic response	Similar response to control	Similar response to control
3 HB ketone bodies	Increased	Similar response to control	Similar response to control
Insulin	Increased	No response	No response
Glucose	Increased	Similar response to control	Similar response to control

* $P < 0.05$ when compared with control values. NEFA, non-esterified fatty acids.

To our knowledge, this increased variability of the NEFA response to meals has not previously been reported. It is also unlikely that this simply reflects the GH surge, as the anticipatory rise in NEFA prior to the second meal does not correlate with the GH peak. A GH-independent effect of ghrelin on lipolysis and glucose metabolism has recently been demonstrated in a recent study by Vestergaard *et al.* (26) who infused ghrelin during a somatostatin clamp. GH-independent effects of ghrelin on body weight and fat mass have also been shown in GH-deficient dwarf rats (27). In our study, ghrelin had a stimulatory effect on insulin in lean individuals and increased post-prandial glucose in all groups, but when corrected for glucose, the insulin/glucose ratio was not altered in any of the groups. Therefore, this is unlikely to explain the biphasic response observed.

Metabolic flexibility, which describes the ability of skeletal muscle and other organs to coordinate carbohydrate and lipid flux during a meal, and is impaired in obese and diabetic individuals, may be a factor here (28). Currently, it is thought that the loss of the cephalic phase of insulin release and alterations in mitochondrial morphology and adipokine release are involved in the underlying mechanisms (29). It is possible that supraphysiological levels of ghrelin also promote metabolic flexibility, enhancing NEFA release in the fasted state, and subsequent meal-induced suppression. This hypothesis is strengthened by our observation in other published work that ghrelin also promotes variability in respiratory quotient in the above subject groups, i.e. ghrelin at pharmacological doses promotes greater oscillations in meal-related substrate utilisation (30). However, we would accept that this is speculative at present and that further data is required to explore this.

Ghrelin also increased ketone body production in all the three groups. This peaked before the first meal and correlated visually with the GH surge during the ghrelin infusion. It is difficult to separate the effects of GH and ghrelin on ketogenesis in this circumstance, but it is noteworthy that previous studies have shown no increase in plasma IGF1 during ghrelin infusion (25, 31). However, unlike NEFA, the ketone response was not biphasic – possibly because ketone body production is slower than that of NEFA during fasting (32).

The main limitation of the infusion study was that the ghrelin infusion achieved supraphysiological plasma levels of total ghrelin. These levels were approximately five- to ten-fold higher than physiological levels after a meal. As we measured total ghrelin and infused acylated ghrelin, the levels of acylated ghrelin may be underestimated compared with physiological release; this can only be speculated as we did not measure acylated ghrelin. In addition, it is thought that acylated ghrelin makes up only around 10% of circulating total ghrelin, and the administration of supraphysiological doses of acylated ghrelin may have overtaken the

influence of non-acylated ghrelin (33, 34). However, it should be noted that recent work has suggested that the ratio of acylated ghrelin to total ghrelin may be altered in obesity and in the metabolic syndrome (35, 36).

The measurement of total ghrelin rather than acylated ghrelin is also a limitation of the fasting study. Liu *et al.* (37) have shown that although acylated ghrelin decreased during fasting in healthy volunteers, des-acylated ghrelin remained elevated at pre-prandial levels. The activity of ghrelin-O-acyltransferase (GOAT or MBOAT4) during fasting should therefore be decreased and indeed Morash *et al.* (38) have confirmed that GOAT mRNA did not increase in fasted mice. Our study showed a trend towards a decrease in total ghrelin in healthy volunteers, which suggest that both acylated and des-acylated ghrelin decreased and a 1:1 ratio was maintained. However, this is speculation as we did not measure acylated ghrelin or GOAT mRNA expression. By comparison, the data by Liu *et al.* (37) shows that total ghrelin did not significantly change during fasting.

An interesting study by Zhao *et al.* (39) has shown that GOAT-deleted mice, after severe prolonged caloric restriction, became hypoglycaemic, and this was likely to be GH mediated as they failed to mount a GH response. Although wild-type mice respond differently to fasting than humans, in that acylated and des-acylated ghrelin steadily increase, this data is important as it suggests that acylated ghrelin may yet have a role in mediating a GH response in conditions of severe calorie restriction. This new rodent data has yet to be confirmed but could have an impact on our conclusions. However, as stated above, we did not measure acylated ghrelin and we accept this as a limitation and that our conclusions are primarily based on our measurements of total ghrelin.

We also accept that the gastrectomy group was significantly older than the other two groups, due to recruitment, and that a lack of correlation between age and fasting ghrelin may be due to small numbers. However, this is unlikely to have influenced the main findings of the study, not least because there was no significant difference in GH release between the gastrectomy group and the control group. Recent research has also suggested that acylated and des-acylated ghrelin bind to various lipoproteins, such as high density lipoprotein (HDL) (40). Our study did not compare these binding proteins among the three groups and we accept that this may have added useful information. We acknowledge that total ghrelin during the fasting study and the infusion study was measured by different assays. However, both of these assays have been used in several published studies, and both use the same competitive RIA principles. It would be unlikely that the use of a single assay for both studies would have altered the conclusions, but again accept this as a limitation.

We can conclude, therefore, that gastrectomy subjects, with low circulating ghrelin, have an intact

metabolic response to short-term fasting. Obese subjects, also with low circulating ghrelin, have an impaired response to fasting, although clearly factors other than ghrelin may be involved. This data collectively suggests a minimal physiological role for ghrelin during fasting, although recent rodent data has challenged this and suggests that acylated ghrelin may have a role in GH response during fasting (39). In contrast, exogenous ghrelin can induce NEFA and ketone body production, suggesting a significant effect on lipid metabolism at supraphysiological doses. We also demonstrate a novel biphasic NEFA response to ghrelin, postulating a significant role for ghrelin in substrate utilisation and metabolic flexibility.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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