European Journal of Endocrinology (2002) **146** 203–207 ISSN 0804-4643

CLINICAL STUDY

Ghrelin drives GH secretion during fasting in man

Alex F Muller[1], Steven W J Lamberts[1], Joop A M J L Janssen[1], Leo J Hofland[1], Peter Van Koetsveld[1], Martin Bidlingmaier[2], Christian J Strasburger[2], Ezio Ghigo[3] and Aart Jan Van der Lely[1]

[1]*Division of Endocrinology, Department of Internal Medicine, Erasmus University Medical Centre, Rotterdam, The Netherlands,* [2]*Division of Endocrinology, Department of Internal Medicine, Med. Klinik Innenstadt, Ludwig-Maximilians University, Munich, Germany and* [3]*Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Turin, Italy*

(Correspondence should be addressed to A F Muller, Department of Internal Medicine, Diakonessenhuis Utrecht, Bosboomstraat 1, 3582 KE, Utrecht, The Netherlands; Email: amuller@diakhuis.nl)

Abstract

Objectives: In humans, fasting leads to elevated serum GH concentrations. Traditionally, changes in hypothalamic GH-releasing hormone and somatostatin release are considered as the main mechanisms that induce this elevated GH secretion during fasting. Ghrelin is an endogenous ligand of the GH secretagogue receptor and is synthesized in the stomach. As ghrelin administration in man stimulates GH release, while serum ghrelin concentrations are elevated during fasting in man, this increase in ghrelin levels might be another mechanism whereby fasting results in stimulation of GH release.
Design and subjects: In ten healthy non-obese males we performed a double-blind placebo-controlled crossover study comparing fasting with and fasting without GH receptor blockade. GH, ghrelin, insulin, glucose and free fatty acids were assessed.
Results: While ghrelin levels do not vary considerably in the fed state, fasting rapidly induced a diurnal rhythm in ghrelin concentrations. These changes in serum ghrelin concentrations during fasting were followed by similar, profound changes in serum GH levels. The rapid development of a diurnal ghrelin rhythm could not be explained by changes in insulin, glucose, or free fatty acid levels. Compared with fasting without pegvisomant, fasting with pegvisomant did not change the ghrelin rhythm.
Conclusions: These data indicate that ghrelin is the main driving force behind the enhanced GH secretion during fasting.

European Journal of Endocrinology **146** 203–207

Introduction

In humans, fasting enhances growth hormone (GH) secretion and amplifies GH rhythm (1). Traditionally hypothalamic GH-releasing hormone and somatostatin release are considered as the mechanisms whereby somatotroph output is regulated (2). Ghrelin is an endogenous ligand of the GH secretagogue (GHS) receptor (GHS-R) and was recently isolated from rat and human stomach, where it is synthesized in a distinct endocrine cell type (3, 4). The gene for ghrelin encodes a 117 amino acid prepro-ghrelin and is expressed not only in the stomach but also in the hypothalamic arcuate nucleus (4). Ghrelin exists in at least two forms, an octanoylated and a non-octanoylated form. At present only octanoylated ghrelin is thought to be biologically active (5–7). I.v. ghrelin administration stimulates GH secretion through the centrally located GHS-R (8–10). Besides this effect on GH secretion a metabolic action of ghrelin is also recognized. In animal studies peripheral ghrelin administration also stimulates food intake, gastric secretion, gastric motility and adiposity (11–13). As ghrelin administration in man strongly stimulates GH release and, at least in animal studies, ghrelin concentrations are elevated during fasting, an increase in ghrelin concentration is another potential mechanism whereby fasting can stimulate GH release (8, 10, 12, 14).

The GHS-R was characterized using a radiolabelled synthetic GHS called MK-0677, and subsequently it was shown that the binding of MK-0677 to the GHS-R could be competitively inhibited by GH-releasing peptide-6 (GHRP-6) (15). Also, the signal transduction pathways for MK-0677 and GHRP-6 both involve phospholipase C, resulting in a rise in inositol triphosphate and intracellular calcium (15). After identification of the GHS-R, a cell line expressing the GHS-R was established and used to identify tissue extracts that could stimulate the GHS-R as monitored by increases in intracellular Ca^{2+} (7). By adding different tissue extracts to such a cell line and monitoring intracellular Ca^{2+} changes, Kojima *et al.* (4) isolated ghrelin

from rat and human stomach. Taken together we can conclude from these data that GHRP-6 and ghrelin bind to the same receptor.

Pegvisomant is a mutated GH molecule that prevents functional dimerization and subsequent activation of the GH receptor (16, 17). In normal subjects and patients with acromegaly pegvisomant effectively blocks GH action and induces a decrease in (free) insulin-like growth factor-I (IGF-I) concentrations (18, 19).

In the present study we have investigated the possible role of ghrelin in the mechanism whereby fasting leads to somatotroph hyperactivity. First, we investigated the effects of fasting on GH and ghrelin concentrations. Secondly, in order to investigate the possible role of some well-known metabolic substrates in the generation of the hypothesized diurnal ghrelin rhythm, we also assessed insulin, glucose and free fatty acid concentrations in serum. Thirdly, to determine the role of GH and its effects via the GH receptor in the regulation of ghrelin secretion during fasting, we investigated if pretreatment with the GH receptor antagonist pegvisomant influenced the effect of fasting on GH and ghrelin levels. Finally, to determine whether an auto-feedback system for ghrelin is operative, and if so to investigate the influence of fasting thereon, we investigated the effect of an i.v. bolus of GHRP-6 (which does not cross-react with the ghrelin assay) on ghrelin levels.

Subjects and methods

Ten healthy male subjects (mean (S.D.) age, 23.4±2.7 years; range 20–28) with a normal body weight (mean (S.D.) body mass index, 21.8±1.8 kg/m^2; range 19.7–25.8) were asked to participate. None of the subjects had a relevant medical history or used medication. The local ethical committee approved the study and all subjects gave written informed consent.

We performed a double-blind placebo-controlled crossover study comparing fasting with and fasting without GH receptor blockade. After an overnight fast, subjects were admitted to the Clinical Research Unit on day 1 at 0730 h. On day 1 and day 4, a GHRP-6 test was performed. At 1800 h on day 1 either pegvisomant 80 mg, as a single s.c. injection, or placebo was administered. A dose of 80 mg was chosen as this dose is capable of reducing the GH concentration-dependent serum IGF-I levels significantly (18), even in acromegalic patients. From 1200 h on day 1 until the end of the study, on day 4 at 2000 h, subjects fasted. Blood was drawn daily at 0800, 1600 and 2400 h. Ghrelin, GH and free IGF-I levels were determined from all samples. Between the study periods there was a wash-out period of 3–7 weeks. GHRP-6 was administered i.v. as a bolus injection of 1 μg/kg. Blood samples were drawn 15 min and immediately before, and 15, 30, 45, 60, 75, 90, 105 and 120 min after GHRP-6 injection. GH levels were determined in all samples. Ghrelin levels were determined from the samples drawn immediately before and 15, 30, 60 and 90 min after GHRP-6 injection. Identical looking vials with GH receptor-antagonist (pegvisomant (Somavert) or placebo were supplied by Sensus Drug Development Corporation, Austin, TX, USA.

GH samples were measured in a two-site immunoassay that does not cross-react with pegvisomant (18). The assay exhibits a lower detection limit of 0.02 μg/l GH, an upper end of the working range of 50 μg/l for 25 μl serum samples, and no cross-reaction with pegvisomant up to a concentration of 50 000 μg/l.

It should be noted that the GH values obtained with this method are approximately 50% of those obtained by conventional RIA methods. The inter-assay coefficient of variation (CV) values were 4.1% at 4.0 μg/l and 3.8% at 20 μg/l. The intra-assay CV values were 3.4% at 0.25 μg/l, 1.9% at 2.5 μg/l and 4.5% at 25 μg/l. Serum free IGF-I was determined with a commercially available IRMA assay (Diagnostic System Laboratories, Webster, TX, USA; intra- and inter-assay CV values 10.3 and 10.7% respectively). Ghrelin was detected with a commercial RIA (Phoenix Pharmaceuticals, Belmont, CA, USA; intra- and interassay CV values 4.5–5.3% and 9.0–13.6% respectively) that uses ^{125}I-labelled bioactive ghrelin as a tracer molecule and a polyclonal antibody raised in rabbits against full-length octanoylated human ghrelin. This assay has no cross-reactivity with other relevant molecules. Insulin was assessed with an RIA (Medgenix Diagnostics, Brussels, Belgium; intra- and inter-assay CV values 13.7 and 8.0% respectively). Glucose was assessed with an automatic hexokinase method (Roche, Almere, The Netherlands). Free fatty acids were determined with an enzymatic colorimetric method (Wako Chemicals GmbH, Neuss, Germany; intra- and inter-assay CV values 1.1 and 4.1% respectively).

Means were compared with the Wilcoxon signed-ranks test. All *P*-values are two-sided, *P*-values <0.05 were considered significant. Area under the curve was calculated using the trapezoidal rule.

Results

Fasting rapidly induced a diurnal ghrelin and GH rhythm (Fig. 1A) that was not seen in the fed state (data not shown). We also assessed insulin, glucose and free fatty acid concentrations in serum. Figure 1B shows that that the gradual changes in serum insulin, glucose and free fatty acid levels are not related in time to the acute changes in systemic ghrelin and GH levels during fasting.

Compared with fasting without pegvisomant, fasting with pegvisomant did not change the ghrelin rhythm (Fig. 2A). However, compared with fasting without the presence of pegvisomant, fasting in combination

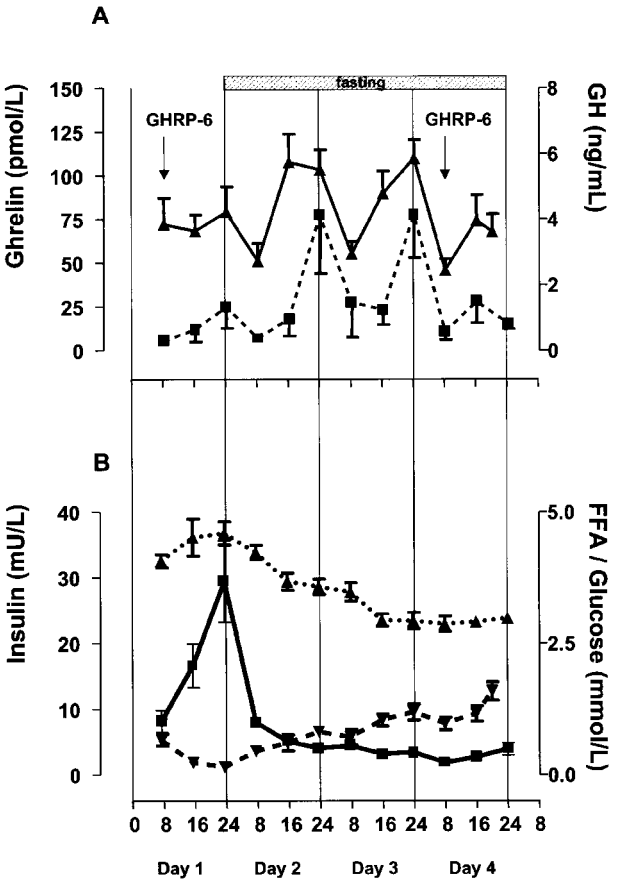

Figure 1 Ghrelin, GH, insulin, glucose and free fatty acid concentrations during fasting and after a bolus injection of GHRP-6. (A) Solid line, ghrelin levels; dotted line, GH levels. (B) Solid line, insulin levels; small dots, glucose levels; large dots, free fatty acid levels. Symbols represent means±S.E.M.

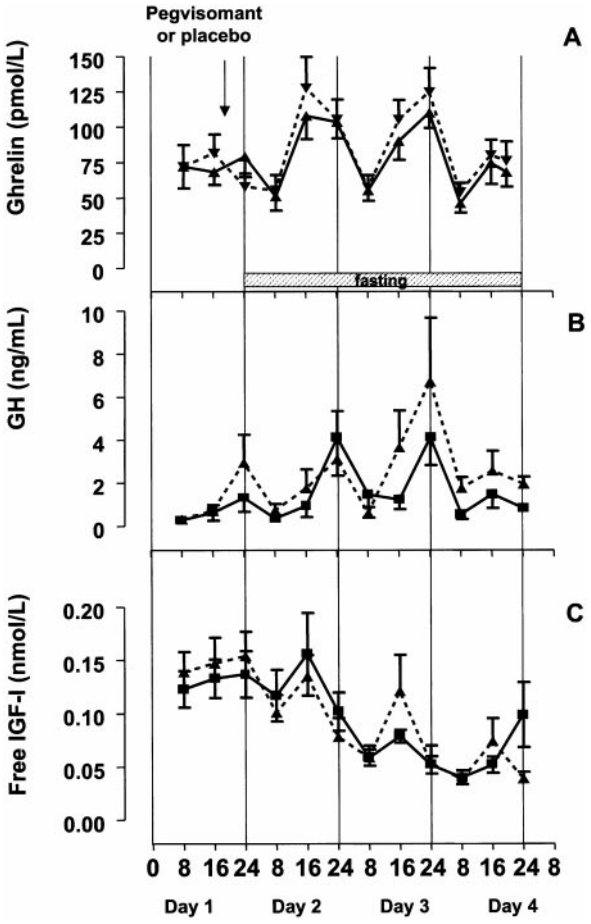

Figure 2 Ghrelin, GH and free IGF-I concentrations during fasting and during fasting in the presence of the GH receptor antagonist pegvisomant. Symbols represent means±S.E.M. Solid line, fasting; dotted line, fasting in the presence of pegvisomant.

with pegvisomant resulted in higher GH concentrations on day 3 (from 0800 h on day 3 to 0800 h on day 4; $P < 0.05$ for difference in area under the curve) (Fig. 2B). In the fasting state, both in the absence and in the presence of pegvisomant, serum free IGF-I levels decreased significantly. However, no additional effect of the presence of a GH receptor antagonist was observed (Fig. 2C).

In all subjects and under all conditions, GHRP-6 administration resulted in a powerful GH release (Fig. 3A). GHRP-6 administration had no acute modifying effects on ghrelin levels (Fig. 3B). However, on study day 4, the third day of fasting, early morning GHRP-6 administration attenuated peak ghrelin levels in the afternoon (Fig. 1A).

Discussion

In the present study we show that fasting rapidly induces an acute and distinct diurnal rhythm in systemic ghrelin concentrations that is not present in the fed state. These changes in serum ghrelin levels during fasting are followed by similar changes in serum GH concentrations, indicating that ghrelin is the driving force of increased GH secretion during fasting. As ghrelin is mainly produced in a distinct endocrine cell of the stomach this implies that the stomach can exert a direct control over the anterior pituitary (3).

In order to investigate the possible role of some well-known metabolic substrates in the generation of this diurnal ghrelin rhythm, we also assessed insulin, glucose and free fatty acid concentrations in serum. Figure 1B clearly shows that that the gradual changes in these metabolic substrates is not related in time to the acute changes in systemic ghrelin and GH levels during fasting. Therefore, the rapid appearance of the observed ghrelin rhythm cannot be explained by acute alterations in insulin, glucose or free fatty acid levels. Interestingly, during fasting pancreatic polypeptide shows a similar diurnal rhythm as ghrelin does

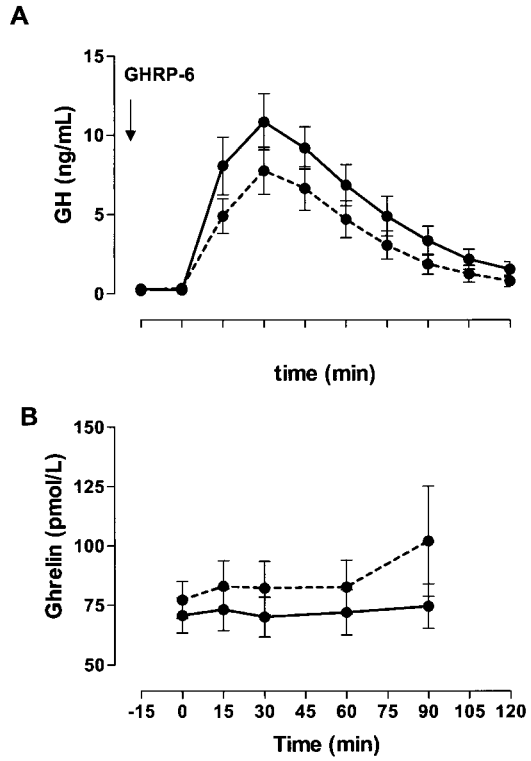

Figure 3 Acute GH and ghrelin response after administration of 1 μg/kg GHRP-6 i.v. (A) Solid line, GH response on the third day of fasting; dotted line, GH response at baseline. (B) Dotted line, ghrelin response at baseline; solid line ghrelin response on the third day of fasting. Symbols represent means±S.E.M.

(20). This opens the possibility that pancreatic polypeptide is responsible for the appearance of a ghrelin rhythm. Since basal pancreatic polypeptide levels are low in human obesity this could also explain why circulating ghrelin levels are decreased in human obesity (21, 22).

It has recently been reported by Cummings *et al.* (23) that there is a large preprandial rise and postprandial fall in plasma ghrelin levels in humans, indicating that ghrelin might be a physiological meal initiatior. We did not observe such a pattern in plasma ghrelin concentrations. However, in the study by Cummings *et al.* subjects were placed for 2 weeks on an outpatient diet and total ingested calories were adjusted to maintain weight stability, whereas in our study subjects received standard meals three times a day only during admission to the Clinical Research Unit and without adjustments to maintain a stable body weight.

Compared with fasting without pegvisomant, pretreatment with pegvisomant resulted in a higher GH output on day 3 (from 0800 h on day 3 to 0800 h on day 4). Apparently, the blockade of the GH receptor induced higher GH levels. Serum free IGF-I levels decreased significantly in the fasting state; this decline was, however, not influenced by the presence or absence of pegvisomant. Thus, disabling the GH–GH receptor signalling system leads to an increase in GH output, while in this same period free IGF-I levels do not change. These data indicate that other factors than free IGF-I, which is considered the most important GH-dependent feedback factor on GH release (24, 25), are responsible for the additional increase in serum GH levels during fasting in the presence of pegvisomant. These data can be taken to indicate an ultra-short feedback loop of GH on its own secretion. Moreover, from these same data we can also conclude that GH does not exert feedback on the production of ghrelin.

An i.v. bolus injection of GHRP-6 had no acute, i.e. within 90 min, effect on ghrelin levels. Notably, on study day 4, the third day of fasting, early morning GHRP-6 administration attenuated peak ghrelin levels in the afternoon. Therefore, we postulate that ghrelin is, at least partially, regulated by a long-loop negative auto-feedback system. However, because we did not include an untreated fasted control group we cannot be entirely sure that the observed attenuation of peak ghrelin levels in the afternoon on the third day of fasting is due to GHRP-6.

In conclusion, we have shown that fasting leads to a diurnal ghrelin rhythm that cannot be explained by changes in insulin, glucose or free fatty acid levels. These changes in serum ghrelin levels during fasting are followed by similar changes in serum GH concentrations, indicating that ghrelin is the driving force of increased GH secretion during fasting. By using the GH receptor antagonist pegvisomant we also provide indirect evidence that these changes in serum ghrelin levels are not regulated by the GH receptor. Finally, we found that the administration of the synthetic GHS, GHRP-6, was followed by a decrease of peak ghrelin levels, but this effect could only be observed after several hours, suggesting that ghrelin concentrations are, at least partially, regulated by a long-loop negative auto-feedback control.

Acknowledgements

A F M was supported by the Dutch Diabetes Fund. The authors would like to thank all the personnel of the Clinical Research Unit of the Erasmus University Medical Centre for their assistance in this study. Sensus Drug Development Corporation, Austin, TX, USA, kindly provided pegvisomant.

References

1 Ho KY, Veldhuis JD, Johnson ML, Furlanetto R, Evans WS, Alberti KG *et al.* Fasting enhances growth hormone secretion and amplifies the complex rhythms of growth hormone secretion in man. *Journal of Clinical Investigation* 1988 **81** 968–975.

2 Giustina A & Veldhuis JD. Pathophysiology of the neuroregulation of growth hormone secretion in experimental animals and the human. *Endocrine Reviews* 1998 **19** 717–797.
3 Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T *et al.* 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* 2000 **141** 4255–4261.
4 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999 **402** 656–660.
5 Bowers CY. Unnatural growth hormone-releasing peptide begets natural ghrelin. *Journal of Clinical Endocrinology and Metabolism* 2001 **86** 1464–1469.
6 Dieguez C & Casanueva FF. Ghrelin: a step forward in the understanding of somatotroph cell function and growth regulation. *European Journal of Endocrinology* 2000 **142** 413–417.
7 Kojima M, Hosoda H, Matsuo H & Kangawa K. Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. *Trends in Endocrinology and Metabolism* 2001 **12** 118–122.
8 Arvat E, Maccario M, Di Vito L, Broglio F, Benso A, Gottero C *et al.* 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* 2001 **86** 1169–1174.
9 Seoane LM, Tovar S, Baldelli R, Arvat E, Ghigo E, Casanueva FF *et al.* Ghrelin elicits a marked stimulatory effect on GH secretion in freely-moving rats. *European Journal of Endocrinology* 2000 **143** R7–R9.
10 Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M *et al.* Ghrelin strongly stimulates growth hormone release in humans. *Journal of Clinical Endocrinology and Metabolism* 2000 **85** 4908–4911.
11 Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z *et al.* Ghrelin stimulates gastric acid secretion and motility in rats. *Biochemical and Biophysical Research Communications* 2000 **276** 905–908.
12 Tschop M, Smiley DL & Heiman ML. Ghrelin induces adiposity in rodents. *Nature* 2000 **407** 908–913.
13 Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S *et al.* The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 2000 **141** 4325–4328.
14 Van Neck JW, Dits NF, Cingel V, Hoppenbrouwers IA, Drop SL & Flyvbjerg A. Dose-response effects of a new growth hormone receptor antagonist (B2036-PEG) on circulating, hepatic and renal expression of the growth hormone/insulin-like growth factor system in adult mice. *Journal of Endocrinology* 2000 **167** 295–303.
15 Smith RG, Van der Ploeg LH, Howard AD, Feighner SD, Cheng K, Hickey GJ *et al.* Peptidomimetic regulation of growth hormone secretion. *Endocrine Reviews* 1997 **18** 621–645.
16 Chen WY, Chen NY, Yun J, Wight DC, Wang XZ, Wagner TE *et al.* Amino acid residues in the third alpha-helix of growth hormone involved in growth promoting activity. *Molecular Endocrinology* 1995 **9** 292–302.
17 Chen WY, Wight DC, Wagner TE & Kopchick JJ. Expression of a mutated bovine growth hormone gene suppresses growth of transgenic mice. *PNAS* 1990 **87** 5061–5065.
18 Thorner MO, Strasburger CJ, Wu Z, Straume M, Bidlingmaier M, Pezzoli SS *et al.* Growth hormone (GH) receptor blockade with a PEG-modified GH (B2036-PEG) lowers serum insulin-like growth factor-I but does not acutely stimulate serum GH. *Journal of Clinical Endocrinology and Metabolism* 1999 **84** 2098–2103.
19 Trainer PJ, Drake WM, Katznelson L, Freda PU, Herman-Bonert V, Van der Lely AJ *et al.* Treatment of acromegaly with the growth hormone-receptor antagonist pegvisomant. *New England Journal of Medicine* 2000 **342** 1171–1177.
20 Villanueva ML, Hedo JA & Marco J. Fluctuations of human pancreatic polypeptide in plasma: effect of normal food ingestion and fasting. *Proceedings of the Society for Experimental Biology and Medicine* 1978 **159** 245–248.
21 Lassmann V, Vague P, Vialettes B & Simon MC. Low plasma levels of pancreatic polypeptide in obesity. *Diabetes* 1980 **29** 428–430.
22 Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E & Heiman ML. Circulating ghrelin levels are decreased in human obesity. *Diabetes* 2001 **50** 707–709.
23 Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE & Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 2001 **50** 1714–1719.
24 Chapman IM, Hartman ML, Pieper KS, Skiles EH, Pezzoli SS, Hintz RL *et al.* Recovery of growth hormone release from suppression by exogenous insulin-like growth factor I (IGF-I): evidence for a suppressive action of free rather than bound IGF-I. *Journal of Clinical Endocrinology and Metabolism* 1998 **83** 2836–2842.
25 Frystyk J, Hojlund K, Christensen M, Beck-Nielsen H & Orskov H. Three days of fasting reduces circulating free but not total IGF-I: a possible role for circulating free IGF in the feedback regulation of GH. *Growth Hormone and IGF Research* 2000 **10** 147 (Abstract).

Received 8 August 2001
Accepted 31 October 2001