Clinical Endocrinology (2004) **61**, 503–509 doi: 10.1111/j.1365-2265.2004.02121.x

Ghrelin secretion is inhibited by glucose load and insulin-induced hypoglycaemia but unaffected by glucagon and arginine in humans

Fabio Broglio*, Cristina Gottero†, Flavia Prodam†, Silvia Destefanis†, Carlotta Gauna*, Elisa Me†, Fabrizio Riganti†, Daniela Vivenza‡, Anna Rapa‡, Valentino Martina†, Emanuela Arvat†, Gianni Bona‡, Aart Jan van der Lely* and Ezio Ghigo†

**Division of Endocrinology, Department of Internal Medicine, Erasmus University of Rotterdam, the Netherlands,* †*Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Turin and* ‡*Unit of Paediatrics, Department of Medical Sciences, 'Piemonte Orientale' University, Novara, Italy*

(Received 30 December 2003; returned for revision 1 February 2004; finally revised 31 May 2004; accepted 18 June 2004)

Summary

OBJECTIVE Circulating ghrelin levels are increased by fasting and decreased by feeding, glucose load, insulin and somatostatin. Whether hyperglycaemia and insulin directly inhibit ghrelin secretion still remains matter of debate. The aim of the present study was therefore to investigate further the regulatory effects of glucose and insulin on ghrelin secretion.

DESIGN AND SUBJECTS We studied the effects of glucose [oral glucose tolerance test (OGTT) 100 g orally], insulin-induced hypoglycaemia [ITT, 0·1 IU/kg insulin intravenously (i.v.)], glucagon (1 mg i.v.), arginine (0·5 mg/kg i.v.) and saline on ghrelin, GH, insulin, glucose and glucagon levels in six normal subjects.

MEASUREMENTS In all the sessions, blood samples were collected every 15 min from 0 up to + 120 min. Ghrelin, GH, insulin, glucagon and glucose levels were assayed at each time point.

RESULTS OGTT increased ($P < 0·01$) glucose and insulin while decreasing ($P < 0·01$) GH and ghrelin levels. ITT increased ($P < 0·01$) GH but decreased ($P < 0·01$) ghrelin levels. Glucagon increased ($P < 0·01$) glucose and insulin without modifying GH and ghrelin. Arginine increased ($P < 0·01$) GH, insulin, glucagon and glucose ($P < 0·05$) but did not affect ghrelin secretion.

CONCLUSIONS Ghrelin secretion in humans is inhibited by OGTT-induced hyperglycaemia and ITT but not by glucagon and arginine, two substances able to increase insulin and glucose levels. These findings question the assumption that glucose and insulin directly regulate ghrelin secretion. On the other hand, ghrelin secretion is not associated with the GH response to ITT or arginine, indicating that the somatotroph response to these stimuli is unlikely to be mediated by ghrelin.

Correspondence: E. Ghigo, Divisione di Endocrinologia, Ospedale Molinette, Corso Dogliotti 14, 10126 Torino, Italy. Tel: +39 011 6963156; Fax: +39 011 6647421; E-mail: ezio.ghigo@unito.it

Ghrelin is a 28-amino acid peptide predominantly produced by the stomach, although it is expressed also in several other tissues including the endocrine pancreas (Kojima *et al*., 2001; Muccioli *et al*., 2002; Broglio *et al*., 2003b; Ukkola, 2003). In its acylated form, ghrelin displays strong GH-releasing activity mediated by the activation of the GH secretagogues receptor type 1a (GHS-R1a); (Kojima *et al*., 2001; Smith *et al*., 2001; Muccioli *et al*., 2002). GHS receptors are concentrated in the hypothalamus–pituitary unit but also distributed in other central and peripheral tissues, including the endocrine pancreas (Kojima *et al*., 2001; Gnanapavan *et al*., 2002; Muccioli *et al*., 2002). However, ghrelin also exerts other endocrine and nonendocrine actions either at the central or peripheral level. In particular, ghrelin has been discovered to be able to exert a strong stimulatory effect on appetite and food intake while modulating energy balance; these actions take place at the central levels where it is likely to be mediated by the neuropeptide Y (NPY) and agouti-related peptide (AGRP) system (Kalra & Kalra, 2003; Chen *et al*., 2004). It has also been shown that ghrelin exerts remarkable metabolic actions at the peripheral level being able to influence endocrine pancreatic function as well as glucose and lipid metabolism (Muccioli *et al*., 2002; Ukkola, 2003).

Circulating ghrelin is mainly in the unacylated form (despite endocrine actions being exerted by its acylated form only) and mostly reflects gastric secretion; in fact, levels are reduced by 70% after gastrectomy as well as after gastric bypass in humans (Ariyasu *et al*., 2001; Cummings *et al*., 2002b; Broglio *et al*., 2003a). Ghrelin secretion occurs in a pulsatile manner without strict correlation with GH levels but with an association to food intake and sleep cycles in rats (Tolle *et al*., 2002). In humans, ghrelin secretion undergoes remarkable variations throughout the

day and ghrelin peaks anticipate food intake, suggesting that the latter is triggered by ghrelin discharge (Cummings *et al*., 2001, 2002b); these findings, however, have not been confirmed by others (Barkan *et al*., 2003).

Ghrelin secretion shows a negative association with body mass index (BMI); circulating total ghrelin levels are increased in anorexia and cachexia but reduced in obesity, notable exception being obese patients with Prader Willi syndrome (Cummings *et al*., 2001, 2002a; Otto *et al*., 2001; Tschop *et al*., 2001b; DelParigi *et al*., 2002; Shiiya *et al*., 2002; Haqq *et al*., 2003; Ukkola, 2003). In agreement with the major link between nutrition and ghrelin, its circulating levels are increased by fasting and energy restriction but decreased by food intake and overfeeding (Ariyasu *et al*., 2001; Cummings *et al*., 2001, 2002b; Tschop *et al*., 2001a; Yoshihara *et al*., 2002). These variations in ghrelin secretion predicted its clear negative association with insulin secretion (Cummings *et al*., 2001; Mohlig *et al*., 2002; Saad *et al*., 2002; Broglio *et al*., 2003b; Flanagan *et al*., 2003; Reimer *et al*., 2003; Schaller *et al*., 2003; Ukkola, 2003).

It has been already demonstrated that ghrelin secretion is reduced by either oral or intravenous glucose load but also, paradoxically, by insulin-induced hypoglycaemia (ITT); (Lucidi *et al*., 2002; Nakagawa *et al*., 2002; Shiiya *et al*., 2002; Flanagan *et al*., 2003). An inhibitory input on ghrelin comes also from the activation of gastric somatostatin receptors as indicated by the finding that the most remarkable inhibition of circulating ghrelin levels is observed under exposure to somatostatin and its analogues (Broglio *et al*., 2002; Norrelund *et al*., 2002; Arosio *et al*., 2003; Barkan *et al*., 2003; Shimada *et al*., 2003).

Regarding the mechanisms underlying the influence of glucose and insulin on ghrelin secretion, it is still a matter of debate if hyperglycaemia and insulin exert direct or indirect actions (Caixas *et al*., 2002; Schaller *et al*., 2003). On the other hand, despite some stimulatory effect of glucagon on ghrelin expression and secretion in animal models (Kishimoto *et al*., 2003; Kamegai *et al*., 2004), the influence of glucagon on circulating ghrelin levels in humans has never been studied. Also the influence of amino acids on ghrelin secretion is still unclear; in fact, some studies reported discrepant results (Lee *et al*., 2002; Groschl *et al*., 2003; Moran *et al*., 2003).

Based on the foregoing, in order to further clarify the mechanisms involved in the regulation of ghrelin secretion in humans, we studied the effects of glucagon and arginine, two well known stimuli of insulin and glucose levels, as well as of oral glucose tolerance test (OGTT)-induced hyperglycaemia and ITT on circulating ghrelin levels in normal young volunteers. In all testing sessions, GH, insulin, glucagon and glucose levels were also assayed.

Research design and methods

Six healthy young male volunteers [age (mean ± SEM): 28·7 ± 2·9 years; BMI: 23·4 ± 0·8 kg/m^2] were studied. All subjects gave their written informed consent to participate in the study which had been approved by an independent ethical committee.

All subjects underwent the following five testing sessions in random order at least 3 days apart:

- saline
- oral glucose load (OGTT, 100 g orally at 0 min)
- ITT [0·1 IU/kg insulin intravenously (i.v.) as a bolus at 0 min]
- intravenous glucagon (1 mg i.v. as a bolus at 0 min)
- i.v. arginine load (ARG, 0·5 mg/kg i.v. as an infusion from time 0 min to +30 min).

After overnight fasting, the tests were begun in the morning at 08·30–09·00 h, 30 min after an indwelling catheter had been placed into an antecubital vein of the forearm kept patent by slow infusion of isotonic saline.

Blood samples were taken every 15 min from 0 up to +120 min. Ghrelin, GH, insulin, glucagon and glucose levels were assayed at each time point in all sessions.

Total plasma ghrelin levels (ng/l) were assayed, after extraction in reverse-phase C18 columns, by a radioimmunometric assay (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA) using ^{125}I-labelled bioactive ghrelin as a tracer and a rabbit polyclonal antibody *vs.* octanoylated and des-octanoylated h-ghrelin (sensitivity: 30 pg/tube, intra-assay coefficient of variation (CV) range: 0·3–10·7%).

Serum GH levels (μg/l: 1 μg/l = 2 mU/l) were measured in duplicate by immunoradiometric assay (IMRA, hGH-CTK IRMA, SORIN Biomedica, Saluggia, Italy). The sensitivity of the assay was 0·15 μg/l. The inter- and intra-assay coefficients of variation were 2·9–4·5% and 2·4–4·0%, respectively.

Serum insulin levels (mU/l) were measured in duplicate by IMRA (INSIK-5, SORIN Biomedica). The sensitivity of insulin assay was 2·5 ± 0·3 mU/l. The inter- and intra-assay coefficients of variation were 6·2–10·8% and 5·5–10·6%, respectively.

Plasma glucagon levels (ng/l) were measured in duplicate by IMRA (Glucagon, Biochem ImmunoSystem, Casalecchio di Reno, Italy). The sensitivity of the assay was 14·5 ng/l. The inter- and intra-assay coefficients of variation were 8·2–9·0% and 8·0–9·5%, respectively. Plasma glucose levels (mmol/l: 1 mg/dl = 0·05551 mmol/l) were measured by gluco-oxidase colourimetric method (GLUCOFIX, by Menarini Diagnostici, Florence, Italy).

All samples from an individual subject were analysed together. The hormonal responses are expressed as absolute values. The statistical analysis was carried out using nonparametric ANOVA (Friedman test) and then Wilcoxon test, as appropriate. The results are expressed as mean ± SEM.

Results

Saline infusion did not modify ghrelin, GH, insulin, glucagon or glucose levels (Figs 1 and 2).

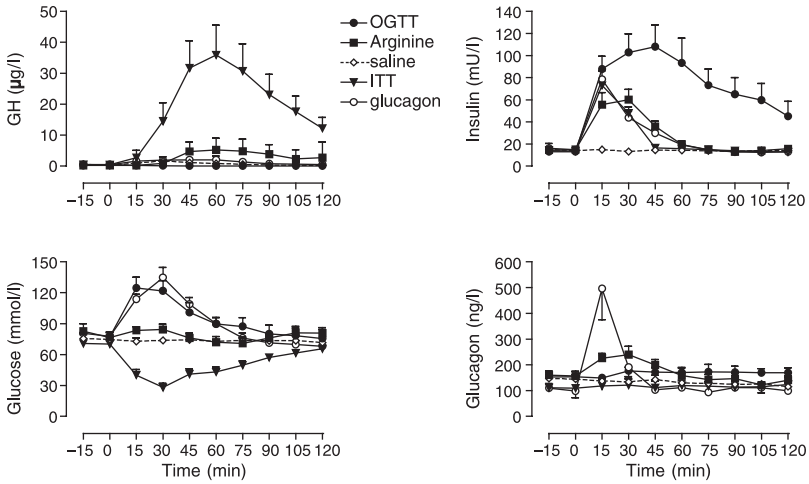

Fig. 1 Mean (± SEM) GH, insulin, glucose and glucagon variation after oral glucose load (●, OGTT, 100 g orally at 0 min), insulin-induced hypoglycaemia (▼, ITT, 0·1 IU/kg insulin i.v. as a bolus at 0 min), intravenous glucagon (○, 1 mg i.v. as a bolus at 0 min), i.v. arginine load (■, 0·5 mg/kg i.v. as an infusion from time 0 min to +30 min) and saline (◇) in six normal young subjects.

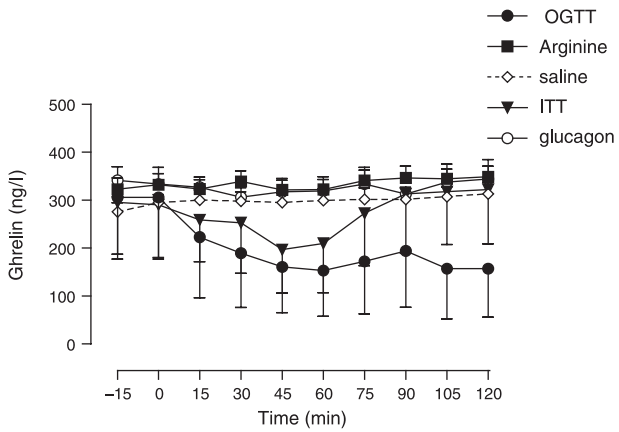

Fig. 2 Mean (± SEM) ghrelin variation after oral glucose load (●, OGTT, 100 g orally at 0 min), insulin-induced hypoglycaemia (▼, ITT, 0·1 IU/kg insulin i.v. as a bolus at 0 min), intravenous glucagon (○, 1 mg i.v. as a bolus at 0 min), i.v. arginine load (■, 0·5 mg/kg i.v. as an infusion from time 0 min to + 30 min) and saline (◇) in six normal young subjects.

Glucose load was followed by decrease in ghrelin levels, with a nadir level occurring at + 60 min (baseline *vs.* nadir: 305·7 ± 128·5 *vs.* 153·0 ± 95·2 ng/l; $P < 0{\cdot}01$; Fig. 1). Oral glucose load obviously increased glucose (baseline *vs.* peak: 4·29 ± 0·25 *vs.* 6·91 ± 0·58 mmol/l; $P < 0{\cdot}01$; peak at time + 15 min) and insulin levels (baseline *vs.* peak: 13·0 ± 2·1 *vs.* 108·0 ± 19·7 mU/l; $P < 0{\cdot}01$; peak at time + 45 min) while significantly decreasing GH levels (baseline *vs.* nadir: 0·5 ± 0·3 *vs.* 0·2 ± 0·0 μg/l; $P < 0{\cdot}01$; nadir at time + 45 min). No significant change in glucagon levels was recorded after OGTT (Figs 1 and 2). Also, ITT was followed by a significant decrease in circulating ghrelin levels with a nadir at + 45 min (baseline *vs.* nadir: 290·5 ± 110·1 *vs.* 197·0 ± 90·6 ng/l; $P < 0{\cdot}01$). ITT was obviously followed by increase in circulating insulin levels and hypoglycaemia, and also induced the expected increase in GH levels (baseline *vs.* peak: 4·8 ± 1·6 *vs.* 35·9 ± 9·7 μg/l; $P < 0{\cdot}01$; peak at time + 60 min). On the other hand, no significant change in glucagon levels was recorded after ITT although a trend toward an increase was observed at the end of the testing session (Figs 1 and 2).

Intravenous glucagon administration was not followed by any change in circulating total ghrelin levels (baseline *vs.* nadir: 333·7 ± 35·2 *vs.* 306·2 ± 19·9 ng/l). Glucagon administration was obviously followed by increase in circulating glucagon levels as well as by the expected increase in glucose (baseline *vs.* peak: 4·30 ± 0·22 *vs.* 7·48 ± 0·54 mmol/l; $P < 0{\cdot}01$; peak at time + 30 min) and insulin levels (baseline *vs.* peak: 14·7 ± 2·4 *vs.* 78·5 ± 7·6 mU/l; $P < 0{\cdot}01$; peak at time + 15 min). No significant change in GH levels was recorded after intravenous glucagon administration (baseline *vs.* peak: 1·2 ± 0·7 *vs.* 2·0 ± 1·2 μg/l; Figs 1 and 2).

Arginine load also did not induce any change in circulating, total ghrelin levels (baseline *vs.* nadir: 332·0 ± 23·3 *vs.* 321·7 ± 20·2 ng/l). As expected, arginine load was followed by an increase in insulin (baseline *vs.* peak: 15·0 ± 1·0 *vs.* 60·1 ± 9·3 mU/l; $P < 0{\cdot}01$; peak at time + 30 min), glucagon levels (baseline *vs.* peak: 156·6 ± 17·3 *vs.* 258·8 ± 26·3 ng/l; $P < 0{\cdot}05$; peak at time + 30 min) and GH levels (baseline *vs.* peak: 0·2 ± 0·1 *vs.* 4·2 ± 1·5 μg/l; $P < 0{\cdot}01$; peak at time + 60 min). A transient increase in glucose levels was also recorded (baseline *vs.* peak: 4·24 ± 0·16 *vs.* 4·68 ± 0·28 mmol/l; $P < 0{\cdot}05$; peak at time + 30 min; Figs 1 and 2).

The evaluation of the hormonal responses to the various tests as area under the curves (AUCs) offered the same results (data not reported). Particularly, the insulin AUCs after OGTT was significantly higher ($P < 0{\cdot}01$) than those recorded after ITT, glucagon and arginine that were not significantly different. Thus the most remarkable inhibitory effect on ghrelin secretion observed after OGTT was associated with the most prominent increase in circulating insulin levels.

The OGTT- and glucagon-induced increases in glucose AUCs were not significantly different. The glucagon AUCs after intravenous injection of glucagon was similar to that recorded after arginine load; both were higher than those after OGTT and ITT, which were not significantly different. The GH AUC recorded after ITT was higher than that after arginine load ($P < 0{\cdot}01$).

Side-effects

No side-effects were recorded after placebo as well as oral glucose load. Arginine administration was followed by transient and mild nausea in two subjects. Similarly, glucagon administration was followed by transient and mild nausea in two subjects. ITT was followed by the classical hypoglycaemic symptoms (hunger, sweating, tremor, restlessness, palpitations), which did not required any medication and disappeared spontaneously.

Discussion

The results of the present study in humans show that ghrelin secretion is not modified by glucagon and arginine, two well-known insulin secretagogues, despite being inhibited by either OGTT-induced hyperglycaemia or ITT. On the other hand, ghrelin secretion is not associated with the GH response to ITT and arginine, making it unlikely that the somatotroph response to these stimuli is mediated by ghrelin.

The natural GH secretagogue ghrelin, as well as leptin, has been proposed as a peripheral hormone signalling to the brain the metabolic balance and contributing to the management of the neuroendocrine and metabolic response to starvation (Muccioli *et al*., 2002; Yoshihara *et al*., 2002; Cummings & Schwartz, 2003). Besides its central orexigenic action, ghrelin also exerts peripheral metabolic actions consistent with the widespread distribution of GHS receptors that are also expressed in the endocrine pancreas (Gnanapavan *et al*., 2002; Muccioli *et al*., 2002). In fact, it has been demonstrated by both animal and human studies that ghrelin is able to affect insulin secretion as well as glucose and lipid metabolism (Muccioli *et al*., 2002; Broglio *et al*., 2003b; Choi *et al*., 2003; Reimer *et al*., 2003; Ukkola, 2003; Zhang *et al*., 2004).

Regarding the regulation of ghrelin secretion, this has been clearly demonstrated to be negatively associated with insulin secretion (Cummings *et al*., 2001; Mohlig *et al*., 2002; Saad *et al*., 2002; Broglio *et al*., 2003b; Flanagan *et al*., 2003; Reimer *et al*., 2003; Schaller *et al*., 2003; Ukkola, 2003) and even to insulin resistance (Lucidi *et al*., 2002; Broglio *et al*., 2003b; Poykko *et al*., 2003; Purnell *et al*., 2003). Indeed, ghrelin secretion had been shown to be reduced by insulin infusion during an euglycaemic clamp (Lucidi *et al*., 2002) as well as by ITT (Lucidi *et al*., 2002; Flanagan *et al*., 2003). However, ghrelin secretion has been also shown paradoxically to be inhibited by either oral and intravenous glucose load (Lucidi *et al*., 2002; Nakagawa *et al*., 2002; Shiiya *et al*., 2002; Flanagan *et al*., 2003). The inhibition of ghrelin secretion following OGTT-induced hyperglycaemia as well as ITT is fully confirmed also by our present study where both the OGTT and ITT reduced circulating ghrelin levels to the same extent (approximately 40%).

Whether insulin and hyperglycaemia directly inhibit ghrelin secretion is, however, still a matter of debate (Caixas *et al*., 2002; Schaller *et al*., 2003). In fact, some authors hypothesized that the inhibitory influence of insulin and hyperglycaemia on ghrelin secretion is more likely reflecting an indirect action (Caixas *et al*., 2002; Schaller *et al*., 2003). To address this point further, we studied the effects of glucagon and arginine, two well-known insulin secretagogues that also induce different degrees of hyperglycaemia (Cryer, 2003), on circulating total ghrelin concentrations. By comparing the effects, if any, of glucagon and arginine with those of OGTT-induced hyperglycaemia and ITT we were expecting to understand better ghrelin regulation, with particular attention to the role of insulin and glycaemia. Moreover, the role of glucagon and amino acids in the regulation of ghrelin secretion is still unclear, at least in humans (Lee *et al*., 2002; Groschl *et al*., 2003; Kishimoto *et al*., 2003; Moran *et al*., 2003; Broglio *et al*., 2004; Kamegai *et al*., 2004).

Our findings show that neither glucagon nor arginine modify ghrelin secretion in humans. We cannot rule out the possibility that increasing the doses of glucagon and arginine would allow us to show that these factors have some influence on ghrelin secretion, although the arginine dose we administered is generally considered maximal (Ghigo *et al*., 1998).

The influence of amino acids on ghrelin secretion was still controversial (Lee *et al*., 2002; Groschl *et al*., 2003; Moran *et al*., 2003); in particular, to our knowledge, no study has previously addressed the role of arginine *per se*, if any, and our study makes it unlikely that it has any relevant influence.

Indeed, glucagon has been proposed as a factor able to stimulate ghrelin expression and secretion based on studies in animal models, i.e. ghrelin expression in the rat stomach and secretion from perfused rat stomach under prolonged exposure to glucagon (Kishimoto *et al*., 2003; Kamegai *et al*., 2004). The absence of any effect of a high dose of glucagon in humans would also reflect the acute administration performed in our present study.

The lack of any effect of glucagon and arginine administration on circulating total ghrelin levels was apparent despite these factors inducing an increase in circulating insulin levels similar to that recorded after ITT. This evidence seems in agreement with other studies questioning the hypothesis that insulin *per se* plays a direct inhibitory role on ghrelin secretion (Lucidi *et al*., 2002; Mohlig *et al*., 2002; Saad *et al*., 2002; Flanagan *et al*., 2003; Murdolo *et al*., 2003). On the other hand, the circulating insulin levels observed after glucagon, arginine and ITT were lower, in terms of AUCs, than that recorded after OGTT; this latter insulin

response to OGTT was associated with an inhibition of ghrelin secretion that seemed more long-lasting. Thus, the possibility that insulin has some direct inhibitory effect on ghrelin synthesis and secretion cannot be definitely ruled out, in agreement with some studies *in vitro* (Kamegai *et al*., 2004; Lippl *et al*., 2004).

It appears more likely that glucose *per se* is not directly regulating ghrelin secretion, in agreement with some previous studies in humans (Lucidi *et al*., 2002; Broglio *et al*., 2003b; Schaller *et al*., 2003) as well as in animals (McCowen *et al*., 2002; Reimer *et al*., 2003). In fact, ghrelin levels are reduced to the same extent after either OGTT-induced hyperglycaemia or ITT (Lucidi *et al*., 2002; Flanagan *et al*., 2003; Gottero *et al*., 2003; Broglio *et al*., 2004; present data). Moreover, ghrelin secretion is inhibited by OGTT but not by glucagon despite the fact that administration of the latter induces a similar increase in plasma glucose levels. Again, it has also been demonstrated that ghrelin secretion is negatively associated with body mass independently of the presence of diabetes mellitus (Shiiya *et al*., 2002).

Another aspect arising from our findings is that the GH response to arginine as well as that to ITT was not associated with any increase in ghrelin secretion, thus suggesting that the somatotroph response to these stimuli is not mediated by ghrelin. That the GH as well as the counter-regulatory response to ITT is coupled to ghrelin decrease and therefore unlikely mediated by this gastric hormone had been already demonstrated (Lucidi *et al*., 2002). Ghrelin secretion is negatively associated to body mass, being increased in anorexia and decreased in obesity, two conditions associated with GH hyper- and hypo-secretion, respectively (Cummings *et al*., 2001; Otto *et al*., 2001; Tschop *et al*., 2001b; Shiiya *et al*., 2002; Ukkola, 2003). Moreover, it has been hypothesized that ghrelin mediates the fasting-induced GH increase (Muller *et al*., 2002); the latter, however, is an experimental model not comparable with ITT (Lucidi *et al*., 2002). Despite this, the physiological role, if any, of ghrelin in the control of GH secretion is still a matter of debate and some studies in humans as well as in animals indicate that ghrelin is unlikely to have a major role (Popovic *et al*., 1995; Tolle *et al*., 2002; Barkan *et al*., 2003). In all, our present data indicate that the GH response to arginine as well as to ITT is unlikely to be mediated by ghrelin. The decrease in ghrelin secretion after OGTT was associated with the well-known inhibitory effect of hyperglycaemia on GH secretion (Valcavi, 1996); once again, however, the decrease in OGTT-induced GH decrease anticipated that in ghrelin levels, making it unlikely that the inhibitory effect of hyperglycaemia on GH secretion is also mediated by ghrelin inhibition.

Finally, intravenous glucagon administration induced the well-known variations in insulin and glucose levels but no change in ghrelin secretion or even in GH secretion. This finding would seem strange considering that intramuscular glucagon is a well-known and reliable provocative stimulus of GH secretion (Rahim *et al*., 1996; Aimaretti *et al*., 2000) widely used for the diagnosis of GH deficiency. However, it has been clearly demonstrated that intravenous glucagon administration is devoid of any stimulatory effect on GH secretion, indicating that glucagon *per se* is not a true GH secretagogue (Ghigo *et al*., 1994).

In conclusion, the results of the present study show that ghrelin secretion in humans is inhibited by OGTT-induced hyperglycaemia and ITT but is unaffected by glucagon and arginine, two substances able to increase insulin and glucose levels. These findings further question the assumption that glucose and insulin directly regulate ghrelin secretion.

Acknowledgements

The present study was supported by Ministero dell'Università e della Ricerca Scientifica (MURST), Eureka (Peptido project 1923), SMEM Foundation and Theratechnologies. The skilful technical assistance of Dr A. Rapa, D. Vivenza, P. van Koetsveld, A. Bertagna, A. Barberis and M. Taliano is acknowledged. The research activity of Fabio Broglio at the Division of Endocrinology and Metabolism of the Erasmus University of Rotterdam is supported by a grant of the GH/IGF-I Society.

References

Aimaretti, G., Baffoni, C., DiVito, L., Bellone, S., Grottoli, S., Maccario, M., Arvat, E., Camanni, F. & Ghigo, E. (2000) Comparison among old and new provocative tests of GH secretion in 178 normal adults. *European Journal of Endocrinology*, **142**, 347–352.

Ariyasu, H., Takaya, K., Tagami, T., Ogawa, Y., Hosoda, K., Akamizu, T., Suda, M., Koh, T., Natsui, K., Toyooka, S., Shirakami, G., Usui, T., Shimatsu, A., Doi, K., Hosoda, H., Kojima, M., Kangawa, K. & Nakao, K. (2001) Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *Journal of Clinical Endocrinology and Metabolism*, **86**, 4753–4758.

Arosio, M., Ronchi, C.L., Gebbia, C., Cappiello, V., Beck-Peccoz, P. & Peracchi, M. (2003) Stimulatory effects of ghrelin on circulating somatostatin and pancreatic polypeptide levels. *Journal of Clinical Endocrinology and Metabolism*, **88**, 701–704.

Barkan, A.L., Dimaraki, E.V., Jessup, S.K., Symons, K.V., Ermolenko, M. & Jaffe, C.A. (2003) Ghrelin secretion in humans is sexually dimorphic, suppressed by somatostatin, and not affected by the ambient growth hormone levels. *Journal of Clinical Endocrinology and Metabolism*, **88**, 2180–2184.

Broglio, F., van Koetsveld, P., Benso, A., Gottero, C., Prodam, F., Papotti, M., Muccioli, G., Gauna, C., Hofland, L., Deghenghi, R., Arvat, E., van der Lely, A.J. & Ghigo, E. (2002) Ghrelin secretion is inhibited by either somatostatin or cortistatin in humans. *Journal of Clinical Endocrinology and Metabolism*, **87**, 4829–4832.

Broglio, F., Benso, A., Gottero, C., Prodam, F., Gauna, C., Filtri, L., Arvat, E., van der Lely, A.J., Deghenghi, R. & Ghigo, E. (2003a) Non-acylated ghrelin does not possess the pituitaric and pancreatic endocrine activity of acylated ghrelin in humans. *Journal of Endocrinological Investigation*, **26**, 192–196.

Broglio, F., Gottero, C., Benso, A., Prodam, F., Volante, M., Destefanis, S., Gauna, C., Muccioli, G., Papotti, M., van der Lely, A.J. & Ghigo, E. (2003b) Ghrelin and the endocrine pancreas. *Endocrine*, **22**, 19–24.

Broglio, F., Prodam, F., Gottero, C., Destefanis, S., Me, E., Riganti, F., Giordano, R., Picu, A., Balbo, M., van der Lely, A.J., Ghigo, E. & Arvat, E. (2004) Ghrelin does not mediate the somatotroph and corticotroph responses to the stimulatory effect of glucagon or insulin-induced hypoglycaemia in humans. *Clinical Endocrinology*, **60**, 699–704.

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

Chen, H.Y., Trumbauer, M.E., Chen, A.S., Weingarth, D.T., Adams, J.R., Frazier, E.G., Shen, Z., Marsh, D.J., Feighner, S.D., Guan, X.M.YeZ., Nargund, R.P., Smith, R.G., Van Der Ploeg, L.H., Howard, A.D., MacNeil, D.J. & Qian, S. (2004) Orexigenic action of peripheral ghrelin is mediated by neuropeptide y and agouti-related protein. *Endocrinology*, **145**, 2607–2612.

Choi, K., Roh, S.G., Hong, Y.H., Shrestha, Y.B., Hishikawa, D., Chen, C., Kojima, M., Kangawa, K. & Sasaki, S. (2003) The role of ghrelin and growth hormone secretagogues receptor on rat adipogenesis. *Endocrinology*, **44**, 754–759.

Cryer, P.E. (2003) Glucose homeostasis and hypoglycemia. In: *Williams Textbook of Endocrinology*, 10th edn. (eds P. R. Larsen, H. M. Kronenberg, S. Melmed & K. S. Polonsky), pp. 1585–1618. Elsevier Science, Philadelphia.

Cummings, D.E. & Schwartz, M.W. (2003) Genetics and pathophysiology of human obesity. *Annual Review of Medicine*, **54**, 453–471.

Cummings, D.E., Purnell, J.Q., Frayo, R.S., Schmidova, K., Wisse, B.E. & Weigle, D.S. (2001) A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes*, **50**, 1714–1719.

Cummings, D.E., Clement, K., Purnell, J.Q., Vaisse, C., Foster, K.E., Frayo, R.S., Schwartz, M.W., Basdevant, A. & Weigle, D.S. (2002a) Elevated plasma ghrelin levels in Prader Willi syndrome. *Nature Medicine*, **8**, 643–644.

Cummings, D.E., Weigle, D.S., Frayo, R.S., Breen, P.A., Ma, M.K., Dellinger, E.P. & Purnell, J.Q. (2002b) Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *New England Journal of Medicine*, **346**, 1623–1630.

DelParigi, A., Tschop, M., Heiman, M.L., Salbe, A.D., Vozarova, B., Sell, S.M., Bunt, J.C. & Tataranni, P.A. (2002) High circulating ghrelin: a potential cause for hyperphagia and obesity in Prader Willi syndrome. *Journal of Clinical Endocrinology and Metabolism*, **87**, 5461–5464.

Flanagan, D.E., Evans, M.L., Monsod, T.P., Rife, F., Heptulla, R.A., Tamborlane, W.V. & Sherwin, R.S. (2003) The influence of insulin on circulating ghrelin. *American Journal of Physiology, Endocrinology and Metabolism*, **284**, E313–E316.

Ghigo, E., Arvat, E., Aimaretti, G., Broglio, F., Giordano, R. & Camanni, F. (1998) Diagnostic and therapeutic uses of growth hormone-releasing substances in adult and elderly subjects. *Baillieres Clinical Endocrinology and Metabolism*, **12**, 341–358.

Ghigo, E., Bartolotta, E., Imperiale, E., Bellone, J., Cardinale, G., Aimaretti, G., Valetto, M.R., Cherubini, V., Maccario, M., Cocchi, D. & Camanni, F. (1994) Glucagon stimulates GH secretion after intramuscular but not intravenous administration. Evidence against the assumption that glucagon *per se* has a GH-releasing activity. *Journal of Endocrinological Investigation*, **17**, 849–854.

Gnanapavan, S., Kola, B., Bustin, S.A., Morris, D.G., McGee, P., Fairclough, P., Bhattacharya, S., Carpenter, R., Grossman, A.B. & Korbonits, M. (2002) The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *Journal of Clinical Endocrinology and Metabolism*, **87**, 2988–2991.

Gottero, C., Bellone, S., Rapa, A., van Koetsveld, P., Vivenza, D., Prodam, F., Benso, A., Destefanis, S., Gauna, C., Bellone, J., Hofland, L., van der Lely, A.J., Bona, G., Ghigo, E. & Broglio, F. (2003) Standard light breakfast inhibits circulating ghrelin level to the same extent of oral glucose load in humans, despite different impact on glucose and insulin levels. *Journal of Endocrinological Investigation*, **26**, 1203–1207.

Groschl, M., Knerr, I., Topf, H.G., Schmid, P., Rascher, W. & Rauh, M. (2003) Endocrine responses to the oral ingestion of a physiological dose of essential amino acids in humans. *Journal of Endocrinology*, **179**, 237–244.

Haqq, A.M., Stadler, D.D., Rosenfeld, R.G., Pratt, K.L., Weigle, D.S., Frayo, R.S., LaFranchi, S.H., Cummings, D.E. & Purnell, J.Q. (2003) Circulating ghrelin levels are suppressed by meals and octreotide therapy in children with Prader Willi syndrome. *Journal of Clinical Endocrinology and Metabolism*, **88**, 3573–3576.

Kalra, S.P. & Kalra, P.S. (2003) Neuropeptide Y: a physiological orexigen modulated by the feedback action of ghrelin and leptin. *Endocrine*, **22**, 49–56.

Kamegai, J., Tamura, H., Shimizu, T., Ishii, S., Sugihara, H. & Oikawa, S. (2004) Effects of insulin, leptin, and glucagon on ghrelin secretion from isolated perfused rat stomach. *Regulation Peptide*, **119**, 77–81.

Kishimoto, M., Okimura, Y., Nakata, H., Kudo, T., Iguchi, G., Takahashi, Y., Kaji, H. & Chihara, K. (2003) Cloning and characterization of the 5(′)-flanking region of the human ghrelin gene. *Biochemical and Biophysical Research Communications*, **305**, 186–192.

Kojima, M., Hosoda, H. & Kangawa, K. (2001) Purification and distribution of ghrelin: the natural endogenous ligand for the growth hormone secretagogue receptor. *Hormone Research*, **5621**, 93–97.

Lee, H.M., Wang, G., Englander, E.W., Kojima, M. & Greeley, G.H. 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.

Lippl, F., Kircher, F., Erdmann, J., Allescher, H.D. & Schusdziarra, V. (2004) Effect of GIP, GLP-1, insulin and gastrin on ghrelin release in the isolated rat stomach. *Regulation Peptide*, **119**, 93–98.

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

McCowen, K.C., Maykel, J.A., Bistrian, B.R. & Ling, P.R. (2002) Circulating ghrelin concentrations are lowered by intravenous glucose or hyperinsulinemic euglycemic conditions in rodents. *Journal of Endocrinology*, **175**, R7–R11.

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

Moran, L.J., Noakes, M., Clifton, P.M., Wittert, G., Tomlinson, L., Galletly, C., Luscombe, N., Tomlinson, L. & Norman, R.J. (2003) Ghrelin and measures of satiety are altered in polycystic ovary syndrome but not differentially affected by diet composition. *Asia Pacific Journal of Clinical Nutrition*, **12**, S52.

Muccioli, G., Tschop, M., Papotti, M., Deghenghi, R., Heiman, M. & Ghigo, E. (2002) Neuroendocrine and peripheral activities of ghrelin: implications in metabolism and obesity. *European Journal of Pharmacology*, **440**, 235–254.

Muller, A.F., Lamberts, S.W., Janssen, J.A., Hofland, L.J., van Koetsveld, P., Bidlingmaier, M., Strasburger, C.J., Ghigo, E. & van der Lely, A.J. (2002) Ghrelin drives GH secretion during fasting in man. *European Journal of Endocrinology*, **146**, 203–207.

Murdolo, G., Lucidi, P., Di Loreto, C., Parlanti, N., De Cicco, A., Fatone, C.,

Fanelli, C.G., Bolli, G.B., Santeusanio, F. & De Feo, P. (2003) Insulin is required for prandial ghrelin suppression in humans. *Diabetes*, **52**, 2923–2927.

Nakagawa, E., Nagaya, N., Okumura, H., Enomoto, M., Oya, H., Ono, F., Hosoda, H., Kojima, M. & Kangawa, K. (2002) Hyperglycaemia suppresses the secretion of ghrelin, a novel growth-hormone-releasing peptide: responses to the intravenous and oral administration of glucose. *Clinical Science*, **103**, 325–328.

Norrelund, H., Hansen, T.K., Orskov, H., Hosoda, H., Kojima, M., Kangawa, K., Weeke, J., Moller, N., Christiansen, J.S. & Jorgensen, J.O. (2002) Ghrelin immunoreactivity in human plasma is suppressed by somatostatin. *Clinical Endocrinology*, **57**, 539–546.

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

Popovic, V., Damjanovic, S., Micic, D., Djurovic, M., Dieguez, C. & Casanueva, F.F. (1995) Blocked growth hormone-releasing peptide (GHRP-6)-induced GH secretion and absence of the synergic action of GHRP-6 plus GH-releasing hormone in patients with hypothalamo-pituitary disconnection: evidence that GHRP-6 main action is exerted at the hypothalamic level. *Journal of Clinical Endocrinology and Metabolism*, **80**, 942–947.

Poykko, S.M., Kellokoski, E., Horkko, S., Kauma, H., Kesaniemi, Y.A. & Ukkola, O. (2003) Low plasma ghrelin is associated with insulin resistance, hypertension, and the prevalence of type 2 diabetes. *Diabetes.*, **52**, 2546–2553.

Purnell, J.Q., Weigle, D.S., Breen, P. & Cummings, D.E. (2003) Ghrelin levels correlate with insulin levels, insulin resistance, and high-density lipoprotein cholesterol, but not with gender, menopausal status, or cortisol levels in humans. *Journal of Clinical Endocrinology and Metabolism*, **88**, 5747–5752.

Rahim, A., Toogood, A.A. & Shalet, S.M. (1996) The assessment of growth hormone status in normal young adult males using a variety of provocative agents. *Clinical Endocrinology*, **45**, 557–562.

Reimer, M.K., Pacini, G. & Ahren, B. (2003) Dose-dependent inhibition by ghrelin of insulin secretion in the mouse. *Endocrinology*, **144**, 916–921.

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

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 cross-over clamp study. *Diabetes*, **52**, 16–20.

Shiiya, T., Nakazato, M., Mizuta, M., Date, Y., Mondal, M.S., 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.

Shimada, M., Date, Y., Mondal, M.S., Toshinai, K., Shimbara, T., Fukunaga, K., Murakami, N., Miyazato, M., Kangawa, K., Yoshimatsu, H., Matsuo, H. & Nakazato, M. (2003) Somatostatin suppresses ghrelin secretion from the rat stomach. *Biochemical and Biophysical Research Communications*, **302**, 520–525.

Smith, R.G., Leonard, R., Bailey, A.R., Palyha, O., Feighner, S., Tan, C., McKee, K.K., Pong, S.S., Griffin, P. & Howard, A. (2001) Growth hormone secretagogue receptor family members and ligands. *Endocrine*, **14**, 9–14.

Tolle, V., Bassant, M.H., Zizzari, P., Poindessous-Jazat, F., Tomasetto, C., Epelbaum, J. & Bluet-Pajot, M.T. (2002) Ultradian rhythmicity of ghrelin secretion in relation with GH, feeding behaviour, and sleep–wake patterns in rats. *Endocrinology*, **143**, 1353–1361.

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

Tschop, M., Weyer, C., Tataranni, P.A., Devanarayan, V., Ravussin, E. & Heiman, M.L. (2001b) Circulating ghrelin levels are decreased in human obesity. *Diabetes*, **50**, 707–709.

Ukkola, O. (2003) Ghrelin and insulin metabolism. *European Journal of Clinical Investigation*, **33**, 183–185.

Valcavi, R. (1996) Oral glucose tolerance test: an inhibitory or a stimulatory input to growth hormone secretion? *Journal of Endocrinological Investigation*, **19**, 253–255.

Yoshihara, F., Kojima, M., Hosoda, H., Nakazato, M. & Kangawa, K. (2002) Ghrelin: a novel peptide for growth hormone release and feeding regulation. *Current Opinion in Clinical Nutrition and Metabolic Care*, **5**, 391–395.

Zhang, W., Zhao, L., Lin, T.R., Chai, B., Fan, Y., Gantz, I. & Mulholland, M.W. (2004) Inhibition of adipogenesis by ghrelin. *Molecular Biology of the Cell*, **15**, 2484–2491.