0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(5):2429–2433
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doi: 10.1210/jc.2003-031517

Acetylcholine Regulates Ghrelin Secretion in Humans

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Ghrelin secretion has been reportedly increased by fasting and energy restriction but decreased by food intake, glucose, insulin, and somatostatin. However, its regulation is still far from clarified. The cholinergic system mediates some ghrelin actions, *e.g.* stimulation of gastric contractility and acid secretion and its orexigenic activity. To clarify whether ghrelin secretion undergoes cholinergic control in humans, we studied the effects of pirenzepine [PZ, 100 mg *per os* (by mouth)], a muscarinic antagonist, or pyridostigmine (PD, 120 mg *per os*), an indirect cholinergic agonist, on ghrelin, GH, insulin, and glucose levels in six normal subjects. PD increased ($P <$ 0.05) GH (change in area under curves, mean ± SEM, 790.9 ± 229.3 μg∗min/liter) but did not modify insulin and glucose levels. PZ did not significantly modify GH, insulin, and glucose levels. Circulating ghrelin levels were increased by PD (11,290.5 ± 6,688.7 pg∗min/ml; $P < 0.05$) and reduced by PZ (−23,205.0 ± 8,959.5 pg∗min/ml; $P < 0.01$). The PD-induced ghrelin peak did not precede that of GH. In conclusion, circulating ghrelin levels in humans are increased and reduced by cholinergic agonists and antagonists, respectively. Thus, ghrelin secretion is under cholinergic, namely muscarinic, control in humans. The variations in circulating ghrelin levels induced by PD and PZ are unlikely to mediate the cholinergic influence on GH secretion. (*J Clin Endocrinol Metab* 89: 2429–2433, 2004)

GHRELIN IS A 28-amino-acid peptide predominantly produced by the stomach, although its expression has also been demonstrated in many other central and peripheral endocrine and nonendocrine tissues (1–4). Acylated ghrelin displays strong GH-releasing activity mediated by the activation of the GH secretagogue receptor 1a (3, 5) that is expressed in the hypothalamus-pituitary unit but also in other central and peripheral tissues (1–3, 5). Ghrelin has other activities including: 1) stimulation of lactotroph and corticotroph secretion and inhibitory influence on the gonadal axis; 2) orexant activity coupled with control of energy expenditure; 3) influence on sleep and behavior; 4) control of gastric motility and acid secretion; 5) influence on the exocrine and endocrine pancreatic function as well as on glucose metabolism; 6) cardiovascular actions; and 7) influence on cell proliferation (3, 4, 6, 7).

Secretion of ghrelin, mostly represented by its unacylated form that has no endocrine activities (8), reflects gastric production and shows remarkable variations throughout the day (9–11) that have not been confirmed by a more recent study (12). Ghrelin secretion seems gender dependent, at least in adulthood when ghrelin levels are higher in women than in men; on the other hand, although a reduction of circulating morning ghrelin levels in aging has been reported, it is still unclear whether age is a critical determinant of ghrelin secretion (12–15). Circulating ghrelin levels are negatively associated with body mass index (BMI); in fact, ghrelin secretion is increased in anorexia and cachexia, reduced in obesity, and normalized by recovery of ideal body weight (11, 14–16). Thus, ghrelin changes in response to variations in the nutritional state are opposite those of leptin, and it has been suggested that both hormones act as signals of the metabolic balance and manage the neuroendocrine and metabolic response to starvation (3, 10).

In humans, circulating ghrelin levels are increased by fasting and energy restriction and decreased by food intake (10, 16), indicating that ghrelin secretion mainly undergoes a metabolic control. Ghrelin secretion is decreased by either iv or oral glucose load as well as during an euglycemic hyperinsulinemic clamp and even during insulin-induced hypoglycemia (4, 17–22). The inhibitory influence of overexposure to insulin on ghrelin secretion agrees with the strong negative association between ghrelin and insulin levels that had been predicted by the negative correlation between ghrelin levels and BMI (10, 13, 15, 20). Whether insulin and glucose *per se* play a direct or indirect inhibitory role on ghrelin secretion is, however, still a matter of debate (23–25).

The most remarkable inhibitory input on ghrelin secretion is represented by the activation of somatostatin (SS) receptors as indicated by evidence that native SS, its natural analog cortistatin, and a synthetic analog such as octreotide lower circulating ghrelin levels in humans (12, 26–28).

The cholinergic system plays a major role in regulating gastroenteropancreatic functions including insulin secretion, as well as hypothalamus-pituitary actions, particularly including GH secretion in humans as well as in animals (29–31). The stimulatory influence of muscarinic receptors on GH secretion is likely to be mediated by the negative modulation of hypothalamic SS release (30, 31); nevertheless, unlike the

Abbreviations: BMI, Body mass index; CV, coefficient(s) of variation; PD, pyridostigmine; PZ, pirenzepine; SS, somatostatin.

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GH response to GHRH, the somatotroph response to ghrelin is basically refractory to both cholinergic agonists and antagonists (32).

A functional relationship between the cholinergic system and ghrelin secretion has already been demonstrated. In rats, truncal vagotomy has been reportedly able to induce mild reduction of stomach ghrelin mRNA levels but also to increase plasma ghrelin levels approximately 3-fold (33). Moreover, it has been shown that acetylcholine mediates some ghrelin actions in rats, *e.g.* stimulation of gastric contractility and acid secretion as well as the orexigenic activity (34–36).

More recently, the peculiar suppression of ghrelin secretion after feeding in sheep is reverted by atropine, a muscarinic receptor antagonist, and hexamethonium, a nicotinic receptor antagonist, respectively, whereas metoclopramide, used as an indirect cholinergic agonist, was devoid of any effect (37). On the other hand, the food deprivation-induced elevation in plasma ghrelin levels in rats has been found abolished by subdiaphragmatic vagotomy and substantially reduced by atropine (38).

To clarify whether and how acetylcholine regulates ghrelin secretion in humans, we studied the effects of either pirenzepine (PZ), a muscarinic antagonist, or pyridostigmine (PD), a cholinesterase inhibitor, on ghrelin secretion in healthy young volunteers. In all the subjects, GH, insulin, and glucose levels after PZ or PD administration were also evaluated.

Subjects and Methods

Six healthy young male volunteers [age (mean ± SEM), 28.9 ± 2.9 yr; BMI, 23.6 ± 0.8 kg/m^2] were studied. All the subjects gave their written informed consent to participate to the study, which had been approved by an independent ethical committee.

All the subjects underwent the following three testing sessions in random order at least 5 d apart: 1) saline; 2) PD [120 mg *per os* (by mouth) at time 0 min]; and 3) PZ (100 mg *per os* at time 0 min).

After overnight fasting, the tests were begun in the morning at 0830–0900 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 at time 0 min and every 15 min from time +45 min up to +180 min. Ghrelin, GH, insulin, and glucose levels were assayed at each time point in all the sessions.

Ghrelin levels (ng/liter) were measured in duplicate, after extraction in reverse phase C18 columns, by RIA (Phoenix Pharmaceutical, Inc., Belmont, CA). Sensitivity was 30 pg/tube; the intraassay coefficient of variation (CV) range was 0.3–10.7%.

GH levels (μg/liter) were measured in duplicate by immunoradiometric assay (hGH-CTK, Sorin Biomedica Cardio, Saluggia, Italy). Sensitivity was 0.15 μg/liter; the inter- and intraassay CV ranges were 2.9–4.5% and 2.4–4.0%, respectively.

Insulin levels (mU/liter) were measured in duplicate by immunoradiometric assay (INSIK-5, Sorin). Sensitivity was 2.5 ± 0.3 mU/liter; the inter- and intraassay CV ranges were 6.2–10.8% and 5.5–10.6%, respectively.

Glucose levels (mg/dl) were measured by glucooxidase colorimetric method (GLUCOFIX, A. Menarini Diagnostics, Florence, Italy).

All samples from an individual subject were analyzed together.

The hormonal responses are expressed as change in area under curves calculated by trapezoidal integration or as percent variations *vs.* baseline. 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

Ghrelin, GH, insulin, and glucose levels did not significantly change during saline administration (Figs. 1 and 2).

PD administration induced a significant increase of GH levels (change in area under curves, 790.9 ± 229.3 μg∗min/liter; $P < 0.05$ *vs.* placebo), with GH peak occurring at the time point +105 min. On the other hand, no significant variation in insulin and glucose levels was recorded after PD administration (Fig 1).

PZ administration did not significantly modify either GH or insulin and glucose levels (Fig. 2).

Ghrelin secretion was modified by either cholinergic enhancement or muscarinic blockade.

PD administration induced a clear increase in ghrelin levels (11,290.5 ± 6,688.7 pg∗min/ml; $P < 0.05$) that reached the peak at time 120 min (about 30% above baseline) (Fig. 1). Thus, the PD-induced ghrelin peak followed that of GH.

On the other hand, PZ administration induced clear inhibition of ghrelin levels (−23,205.0 ± 8,959.5 pg∗min/ml; $P < 0.01$). Ghrelin levels underwent a progressive decrease from time +45 min up to the end of the testing session (Fig. 2). The

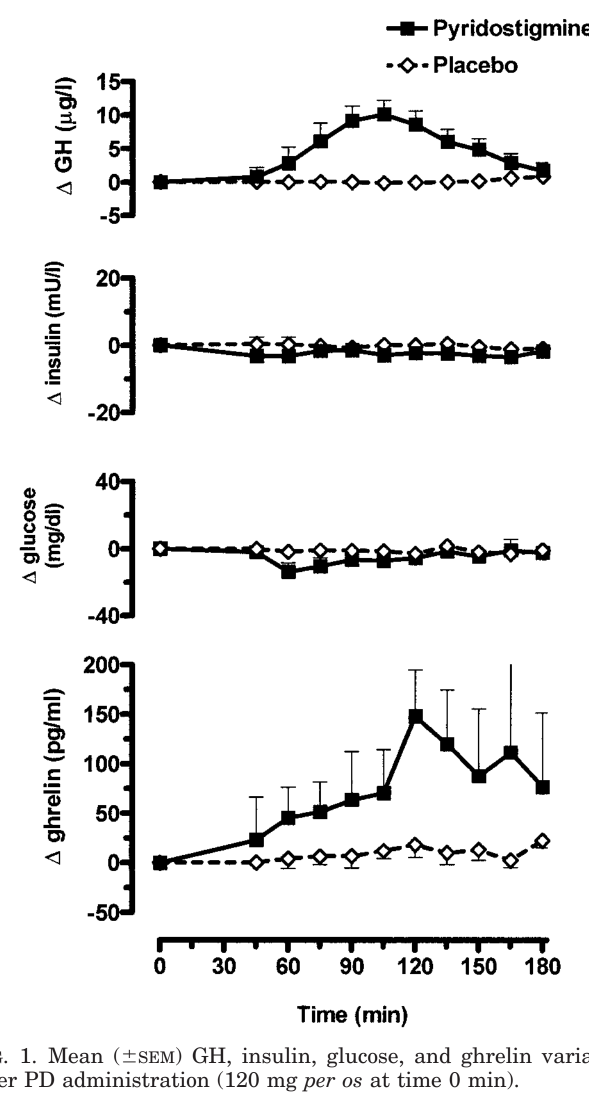

FIG. 1. Mean (±SEM) GH, insulin, glucose, and ghrelin variations after PD administration (120 mg *per os* at time 0 min).

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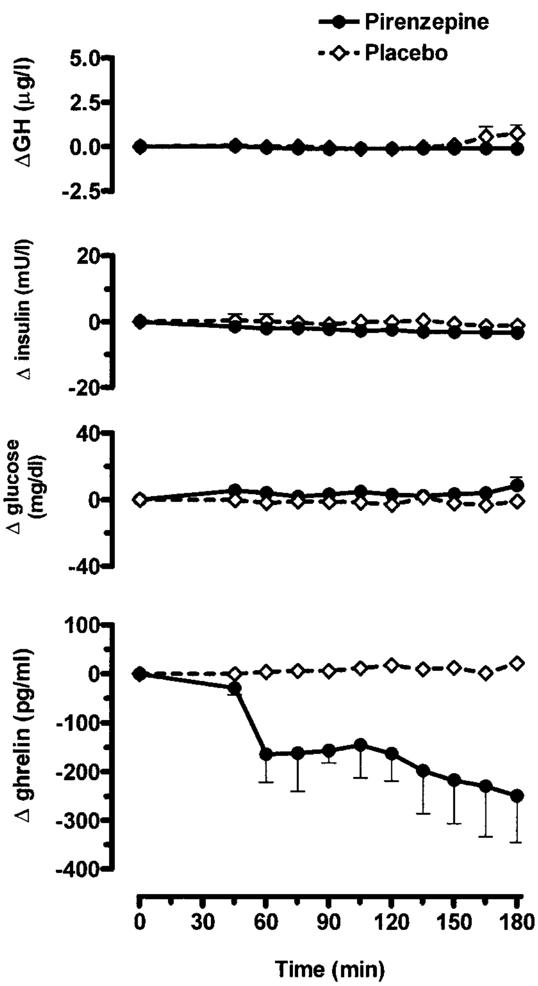

FIG. 2. Mean (±SEM) GH, insulin, glucose, and ghrelin variations after PZ administration (100 mg *per os* at time 0 min).

decrease in circulating ghrelin levels after PZ was 37% below baseline.

Side effects

In three subjects, after PD administration, mild abdominal pain and muscle fasciculation were observed. In two subjects, after PZ administration, mouth dryness and impairment of visual accommodation were observed.

Discussion

The present study demonstrates that ghrelin secretion in humans is stimulated by a cholinergic agonist such as PD and inhibited by a muscarinic receptor blocker such as PZ. Neither PD- nor PZ-induced variations in circulating ghrelin levels are associated with changes in insulin and glucose levels. Moreover, the PD-induced ghrelin increase does not precede the GH response to the enhancement of the cholinergic tone elicited by the cholinesterase inhibitor.

Circulating ghrelin levels mostly reflect gastric secretion, as demonstrated by evidence that they are reduced by 80% after gastrectomy (16). It has been clearly demonstrated that ghrelin secretion is increased by fasting and energy restriction and reduced by food intake as well as by glucose, insulin, and SS (3, 4, 7, 10, 12, 14, 17, 21, 22, 26–28). Recent studies suggested also that peptide YY and oxyntomodulin would exert an inhibitory action on ghrelin secretion because its circulating levels have been found reduced under chronic treatment with peptide YY (3–36) and oxyntomodulin in humans (39, 40). Thus, although we know of several factors able to inhibit ghrelin secretion, so far, we know only conditions (*e.g.* fasting and energy restriction) but not single factors able to increase ghrelin levels.

Acetylcholine is a neurotransmitter playing a major role in the control of gastroenteropancreatic exocrine and endocrine secretions (29, 41). Moreover, it had been recently emphasized that the cholinergic system and ghrelin secretion are likely to be linked by a functional relationship (33, 37).

Some major ghrelin actions, such as the stimulation of gastric contractility and acid secretion as well as the orexigenic activity, are mediated by the cholinergic system in rats (34–36, 38). On the other hand, in humans, the endocrine actions of acylated ghrelin (influence on GH, prolactin, ACTH, and insulin) are refractory to both cholinergic agonists and antagonists (32).

Regarding the potential role of acetylcholine in the regulation of ghrelin secretion, mild inhibition of stomach ghrelin mRNA levels, coupled with an increase in plasma ghrelin levels, have been observed after truncal vagotomy in rats (33). In sheep, the food-induced decrease in ghrelin levels has been found reverted by atropine, a muscarinic receptor antagonist, and hexamethonium, a nicotinic receptor antagonist, respectively; in the same study, metoclopramide, used as an indirect cholinergic agonist, was devoid of any effect (37).

The present study shows that muscarinic antagonism by PZ inhibits, whereas cholinergic enhancement by PD augments ghrelin secretion in humans. Thus, these findings are the first to demonstrate that ghrelin secretion in humans is under major stimulatory control by acetylcholine, mainly via a muscarinic mechanism.

Our present data disagree with those in sheep (see above), indicating an inhibitory role of acetylcholine on ghrelin secretion. However, our findings fit well with other data (33) and particularly with recent evidence that a food-induced increase in ghrelin secretion in rats is completely prevented by supradiaphragmatic vagotomy and substantially reduced by atropine (38). Species-specific differences would explain this discrepancy; in fact, peculiar cholinergic regulation has been demonstrated already in sheep that also shows peculiar ghrelin response to energy restriction (37).

Thus, it seems clear that, at least in humans as well as in rats, acetylcholine plays a stimulatory role on ghrelin secretion.

In our study, the degree of ghrelin inhibition observed under muscarinic blockade by PZ was remarkable (~30%) and similar to that recorded after hyperglycemia or during euglycemic-hyperinsulinemic clamp (18, 19, 21, 22). The most important inhibitory influence on ghrelin secretion reported so far is, however, that exerted by the activation of SS receptors by native SS as well as by its natural and synthetic analogs (12, 26–28, 42). The inhibitory somatostatinergic influence is likely to take place directly at the gastric level,

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where SS receptors have been demonstrated (43). On the other hand, SS expression and release are under stimulatory influence by ghrelin (44), suggesting a feedback link between these two hormones. Because acetylcholine and SS are, in turn, linked by a functional feedback link in which acetylcholine negatively modulates SS secretion (29–31), this picture suggests that the cholinergic influence on ghrelin secretion would be theoretically mediated by SS inhibition.

Although ghrelin is reportedly likely to be involved in the control of insulin secretion and glucose metabolism (3, 4, 33, 45), in the present study, ghrelin increase and decrease triggered by PD and PZ, respectively, were not associated with any change in insulin and glucose levels. The influence of ghrelin on insulin and glucose levels has generally been observed after acute administration of acylated ghrelin at a pharmacological dose (45). Here, we measured total circulating ghrelin levels; thus, we are not able to distinguish between the acylated and unacylated circulating forms (46) and cannot speculate on the levels of octanoylated ghrelin that is considered the biologically active form (2, 8). It remains that, within the range of variations in circulating ghrelin levels induced by cholinergic enhancement or blockade, there was no association with insulin levels that have been demonstrated to be negatively correlated with ghrelin secretion (4, 14). On the other hand, the positive influence of acetylcholine on insulin secretion mostly takes place in terms of amplification of the insulin response to secretagogues or has been described as a direct action on pancreatic β-cells (29). Thus, the lack of any significant insulin response to cholinergic enhancement by PD or blockade by PZ in the present study agrees with other reports in literature (29).

Finally, we confirm the well-known stimulatory effect of PD on GH secretion (47); this GH increase was not preceded by the PD-induced increase in ghrelin levels. This finding would suggest that the stimulatory effect of acetylcholine on somatotroph function is not mediated by ghrelin and remains better explained by the negative modulation of hypothalamic SS release (30, 31). Indeed, in the present study, PZ did not significantly reduce basal GH secretion, but this agrees with studies showing that M1 muscarinic blockade inhibits activated GH secretion only (30, 31).

The physiological relevance of a functional link between ghrelin and somatotroph secretion is still unclear. On one hand, a positive association between GH and ghrelin secretion in obesity and anorexia and evidence that the fasting-induced GH increase is preceded by an increase in ghrelin secretion have been reported (3, 22, 48). On the other hand, insulin-induced hypoglycemia as well as arginine stimulate GH secretion, despite any stimulatory effect on ghrelin levels (20, 49). Daily ghrelin secretion is not strictly correlated with GH secretion and is unchanged during infusion of a GHRH antagonist that markedly inhibits GH secretion (12). Moreover, the ghrelin knockout mouse is not dwarf and not anorectic as well (50).

Once again, it must be emphasized that our present data describe the response of total circulating ghrelin levels to cholinergic manipulations. In the absence of any information about the levels of the acylated active form of ghrelin, we cannot definitely rule out the possibility that it is, at least partially, involved in the mechanisms underlying the stimulatory effect of acetylcholine on somatotroph secretion.

In conclusion, this study for the first time demonstrates that ghrelin secretion in humans is under the stimulatory control of the cholinergic, namely muscarinic, receptors. Acetylcholine is therefore the first stimulatory neurotransmitter shown to play a stimulatory role on ghrelin secretion in humans.

Acknowledgments

The skillful technical assistance of Mrs. M. Taliano and A. Bertagna is acknowledged.

Received August 29, 2003. Accepted January 27, 2004.

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This work was supported by Eureka Project (Peptido 1923), Ministero dell'Università e della Ricerca Scientifica, University of Turin, and Fondazione per lo Studio delle Mulattie Endocrino-Metaboliche. The research activity of F.B. at the Division of Endocrinology and Metabolism of the Erasmus University of Rotterdam is supported by a grant from the GH/IGF-I Society.

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