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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*

EMANUELA ARVAT, MAURO MACCARIO, LIDIA DI VITO, FABIO BROGLIO, ANDREA BENSO, CRISTINA GOTTERO, MAURO PAPOTTI, GIAMPIERO MUCCIOLI, CARLOS DIEGUEZ, FELIPE F. CASANUEVA, ROMANO DEGHENGHI, FRANCO CAMANNI, AND EZIO GHIGO

Division of Endocrinology and Metabolism, Department of Internal Medicine (E.A., M.M., L.D.V., F.B., A.B., C.G., F.C., E.G.), Department of Biomedical Sciences and Oncology (M.P.), Department of Anatomy, Pharmacology and Forensic Medicine (G.M.), University of Turin, 10126 Turin, Italy; Department of Physiology (C.D.) and Department of Medicine (F.F.C.), Faculty of Medicine, Santiago de Compostela University, Santiago de Compostela E-15780, Spain; and Europeptides (R.D.), Argenteuil 95108 Cedex, France

ABSTRACT

An endogenous ligand for the GH secretagogue-receptor (GHS-receptor) has recently been isolated, from both the rat and the human stomach, and named ghrelin. It is a 28-amino-acid peptide showing a unique structure with an n-octanoyl ester at its third serine residue, which is essential for its potent stimulatory activity on somatotroph secretion. In fact, it has been demonstrated that ghrelin specifically stimulates GH secretion from both rat pituitary cells in culture and rats *in vivo*. The aim of the present study was to test the GH-releasing activity of ghrelin in humans and to compare it with that of GHRH and hexarelin (HEX), a nonnatural peptidyl GHS, which possesses strong GH-releasing activity but also significantly stimulates PRL, ACTH, and cortisol secretion. To clarify the mechanisms of action underlying the GH-releasing activity of ghrelin in humans, its interaction with GHRH and HEX was also studied. Seven normal young volunteers (7 men; 24–32 yr old; body mass index, 20–24 kg/m^2) were studied. All subjects underwent the administration of ghrelin, HEX, and GHRH-29 (1.0 μg/kg iv at 0 min) as well as placebo (2 mL isotonic saline iv at 0 min). Six subjects also underwent the combined administration of ghrelin and GHRH or HEX. Blood samples were taken every 15 min from -15 up to $+180$ min. GH levels were assayed at each time point in all sessions; PRL, ACTH, cortisol, and aldosterone levels were also assayed after administration of ghrelin and/or HEX. Ghrelin administration induced a prompt and marked increase in circulating GH levels (Cmax, mean $\pm$ SEM, 92.1 $\pm$ 16.7 μg/L; area under the curve, 1894.9 $\pm$ 347.8 μg/L·h). The GH response to ghrelin was clearly higher ($P < 0.01$) than the one recorded after GHRH (26.7 $\pm$ 8.7 μg/L; 619.6 $\pm$ 174.4 μg/L·h) and even significantly higher ($P < 0.05$) than after HEX (68.4 $\pm$ 14.7 μg/L; 1546.9 $\pm$ 380.0 μg/L·h). Ghrelin administration also induced an increase in PRL, ACTH, and cortisol levels; these responses were higher ($P < 0.05$) than those elicited by HEX. A significant increase in aldosterone levels was recorded after ghrelin but not after HEX. The endocrine responses to ghrelin were not modified by the coadministration of HEX. On the other hand, the coadministration of ghrelin and GHRH had a real synergistical effect ($P < 0.05$) on GH secretion (133.6 $\pm$ 22.5 μg/L; 3374.3 $\pm$ 617.3 μg/L·h). In conclusion, ghrelin, a natural ligand of GHS-receptor, exerts a strong stimulatory effect on GH secretion in humans, releasing more GH than GHRH and even more than a nonnatural GHS such as HEX. Ghrelin, as well as HEX, also stimulates lactotroph and corticotroph secretion. Ghrelin shows no interaction with HEX, whereas it has a synergistical effect with GHRH on GH secretion. Thus, ghrelin is a new hormone playing a major role in the control of somatotroph secretion in humans, and its effects are imitated by nonnatural GHS. (*J Clin Endocrinol Metab* **86:** 1169–1174, 2001)

IT HAD been assumed for many years that GH secretion was mainly regulated by two hypothalamic hypophysiotropic neurohormones, GHRH and somatostatin, although the important role of neurotransmitters, peripheral hormones, and metabolic fuels had also been recognized (1, 2).

The existence of another major, unknown factor involved in the control of somatotroph function had been hypothesized, based on the evidence that synthetic, nonnatural peptidyl, and nonpeptidyl molecules [named GH secretagogues (GHSs)] possess strong GH-releasing activity acting on the pituitary, but mainly on the hypothalamus, where specific receptors [GHS receptors (GHS-Rs)] are present (3–8). The endogenous ligand of this so-called orphan receptor was thought to be another hypothalamic neuropeptide (8).

Surprisingly, an endogenous ligand for the GHS-R has recently been purified, from both the rat and the human stomach, and named ghrelin (9). It is a 28-amino-acid peptide showing a unique structure with an n-octanoyl ester at its third serine residue, which is essential for its potent stimulatory activity on somatotroph secretion. In fact, it has been shown that ghrelin specifically stimulates GH secretion from both rat pituitary cells in culture and rats *in vivo* (9). More

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Address all correspondence and requests for reprints to: E. Ghigo, M.D., Divisione di Endocrinologia, Ospedale Molinette, Corso Dogliotti 14, 10126 Torino, Italy. E-mail: ezio.ghigo@unito.it.

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recently, another ligand, showing the same activity, has been purified from the rat stomach; it is a 27-amino-acid peptide named des-Gln14-ghrelin, the sequence of which is identical to ghrelin except for one glutamine (10). Therefore, there is evidence that GH release is regulated by two gastric peptides secreted by endocrine cells, even if brain ghrelin immunoreactive neurons have also been localized in the hypothalamic arcuate nucleus (9).

Human ghrelin is homologous to rat ghrelin, apart from two amino acids, and circulates in human blood at considerable plasma concentrations (9, 11).

We have recently shown that human ghrelin inhibits the binding of ^{125}I-Tyr-Ala-hexarelin from pituitary and hypothalamic membranes in humans (12); and in a preliminary study, we demonstrated, in a few normal subjects, that the administration of 1.0 μg/kg human ghrelin elicits a strong increase in circulating GH levels (13).

The aim of the present study was to compare, in humans, the GH-releasing activity of ghrelin with that of either GHRH or hexarelin (HEX), a nonnatural, synthetic hexapeptide belonging to the GHS family (5). Because nonnatural GHSs generally possess strong GH-releasing activity, but also a significant stimulatory effect on PRL, ACTH, and cortisol secretion (4, 14), the effect of ghrelin administration on these hormones was also verified and compared with that of HEX. To clarify the mechanisms of action underlying the endocrine activity of ghrelin in humans, its interaction with GHRH and HEX was also studied.

Materials and Methods

Peptides

Vials containing 100 μg lyophilized human ghrelin (Mwt 3370.5) were kindly provided by Europeptides (Argenteuil, France). Human ghrelin was prepared under Good Manufactory Practice conditions by Neosystem (Strasbourg, France) and purified to more than 98%. Sterility was guaranteed by filtration on individual Millipore Corp. (Bedford, MA) filters at injection. Absorption of ghrelin on filters was determined experimentally and found not to exceed 10%.

Vials containing 100 μg lyophilized HEX (Mwt 887.06) were kindly provided by Europeptides.

Vials containing 50 μg GHRH-29 (Mwt 5039.7) were kindly provided by Serono (Roma, Italy).

Subjects and study protocol

Seven healthy young male volunteers (age, mean ± SEM, 28.6 ± 2.9 yr; body mass index, 22.1 ± 0.8 kg/m^2) were studied. All subjects gave their written informed consent to participate in the study, which had previously been approved by the independent Ethical Committee of the University of Turin.

All subjects underwent the following four testing sessions, in single-blind, random order, and at least 3 days apart: 1) placebo (2 mL isotonic saline iv at 0 min); 2) ghrelin (1.0 μg/kg iv at 0 min); 3) HEX (1.0 μg/kg iv at 0 min); and 4) GHRH-29 (1.0 μg/kg iv at 0 min). Six subjects also underwent two further testing sessions in which they received the combined administration of ghrelin and HEX or ghrelin and GHRH.

After overnight fasting, the tests were begun at 0830–0900 h, 30 min after an indwelling catheter had been inserted in an antecubital vein of the forearm, kept patent by slow infusion of isotonic saline.

Blood samples were taken every 15 min, from −15 up to +180 min. GH levels were assayed at each time point in all sessions; PRL, ACTH, cortisol, and aldosterone levels were also assayed after ghrelin and/or HEX administration.

Serum GH levels (μg/L) were measured in duplicate by immunoradiometric assay (hGH-CTK IRMA, SORIN, Saluggia, Italy). The sensitivity of the assay was 0.15 μg/L. The inter- and intraassay variation coefficients were 2.9–4.5% and 2.4–4.0%, respectively.

Serum PRL levels (μg/L) were measured in duplicate by immunoradiometric assay (PRL-CTK, IRMA, SORIN). The sensitivity of the assay was 0.15 μg/L. The inter- and intraassay variation coefficients ranged between 3.9 and 6.8% and between 3.3 and 7.5%, respectively.

Plasma ACTH levels (pmol/L) were measured in duplicate by immunoradiometric assay (Allegro HS-ACTH, Nichols Institute Diagnostics, San Juan Capistrano, CA). The sensitivity of the assay was 0.22 pmol/L. The inter− and intraassay variation coefficients ranged between 6.9 and 8.9% and between 1.1 and 3.0%, respectively.

Serum cortisol levels (nmol/L) were measured in duplicate by RIA (CORT-CTK 125, IRMA, SORIN). The sensitivity of the assay was 11.0 nmol/L. The inter- and intraassay variation coefficients ranged between 6.6 and 7.5% and between 3.8 and 6.6%, respectively.

Serum aldosterone levels (pmol/L) were measured in duplicate by RIA (CORT-CTK 125, IRMA, SORIN). The sensitivity of the assay was 16.2 pmol/L. The inter- and intraassay variation coefficients ranged between 6.6 and 7.5% and between 3.8 and 6.6%, respectively.

All samples from an individual subject were analyzed together.

The hormonal responses are expressed as mean ± SEM absolute values and areas under curves (AUCs, from 0–180 min), calculated by trapezoidal integration.

The statistical analysis was carried out using nonparametric ANOVA (Friedman test) and later by a Wilcoxon matched-pairs test.

Results

Basal GH levels were similar in all sessions. Similarly, basal PRL, ACTH, cortisol, and aldosterone levels, before the administration of ghrelin, HEX, or placebo, were similar.

Placebo administration did not modify GH levels (Fig. 1).

Ghrelin induced a prompt and marked increase in circulating GH levels. In fact, the maximal mean GH concentration after ghrelin injection (Cmax, 92.1 ± 16.7 μg/L, $P < 0.01$) was recorded with a mean timing of occurrence (Tmax) of 30.0 ± 3.3 min, whereas the GH AUC after ghrelin administration was 1894.9 ± 347.8 μg/L·h (Fig. 1).

The GH response to ghrelin was clearly higher ($P < 0.01$) than the one recorded after GHRH (Cmax, 26.7 ± 8.7 μg/L; AUC, 619.6 ± 174.4 μg/L·h; Tmax, 25.7 ± 4.3 min) and even significantly higher ($P < 0.05$) than that after HEX (Cmax, 68.4 ± 14.7 μg/L; AUC, 1546.9 ± 380.0 μg/L·h; Tmax, 30.0 ± 3.3 min) (Fig. 1).

Placebo administration did not modify PRL and aldosterone levels, whereas a trend toward decrease in ACTH and cortisol levels, after its administration, was present (Fig. 2).

Ghrelin administration also induced an increase in PRL (Cmax, 14.9 ± 2.2 μg/L; AUC, 545.0 ± 43.1 μg/L·h; Tmax, 17.5 ± 2.5 min), ACTH (Cmax, 20.3 ± 4.5 pmol/L; AUC, 407.1 ± 71.9 pmol/L·h; Tmax, 15.0 ± 0.0 min), cortisol (Cmax, 503.2 ± 43.5 nmol/L; AUC, 20266.5 ± 2559.8 nmol/L·h; Tmax, 34.3 ± 4.3 min), and aldosterone (Cmax, 514.0 ± 109.2 pmol/L; AUC, 22206.9 ± 4407.6 pmol/L·h; Tmax, 35.0 ± 9.2 min) levels ($P < 0.05$) (Fig. 2).

The PRL response to ghrelin was higher ($P < 0.05$) than the one recorded after HEX (Cmax, 10.9 ± 0.9 μg/L; AUC, 438.2 ± 31.4 μg/L·h; Tmax, 17.5 ± 2.5 min) administration (Fig 2). Similarly, both ACTH and cortisol responses to ghrelin were higher ($P < 0.02$) than those recorded after HEX (Cmax: 10.2 ± 2.5 pmol/L, AUC: 264.5 ± 30.6 pmol/L·h, Tmax: 15.0 ± 0.0 min; and Cmax: 394.8 ± 30.0 nmol/L, AUC: 15228.8 ± 1414.8 nmol/L·h, Tmax: 22.5 ± 3.4 min) (Fig 2). On the other hand, differently from ghrelin, HEX did not stimulate aldosterone release (Fig. 2).

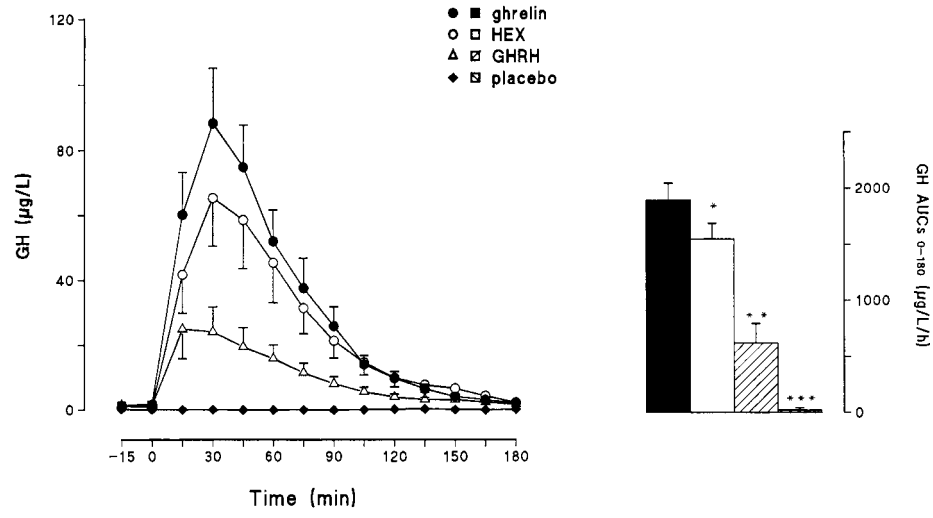

FIG. 1. Mean (± SEM) GH curve responses (*left*) and AUCs (*right*) after ghrelin (1.0 μg/kg), HEX (1.0 μg/kg), GHRH (1.0 μg/kg), or placebo in normal subjects. *, $P < 0.05$ *vs.* ghrelin; **, $P < 0.02$ *vs.* HEX and ghrelin; ***, $P < 0.01$ *vs.* GHRH, HEX, and ghrelin.

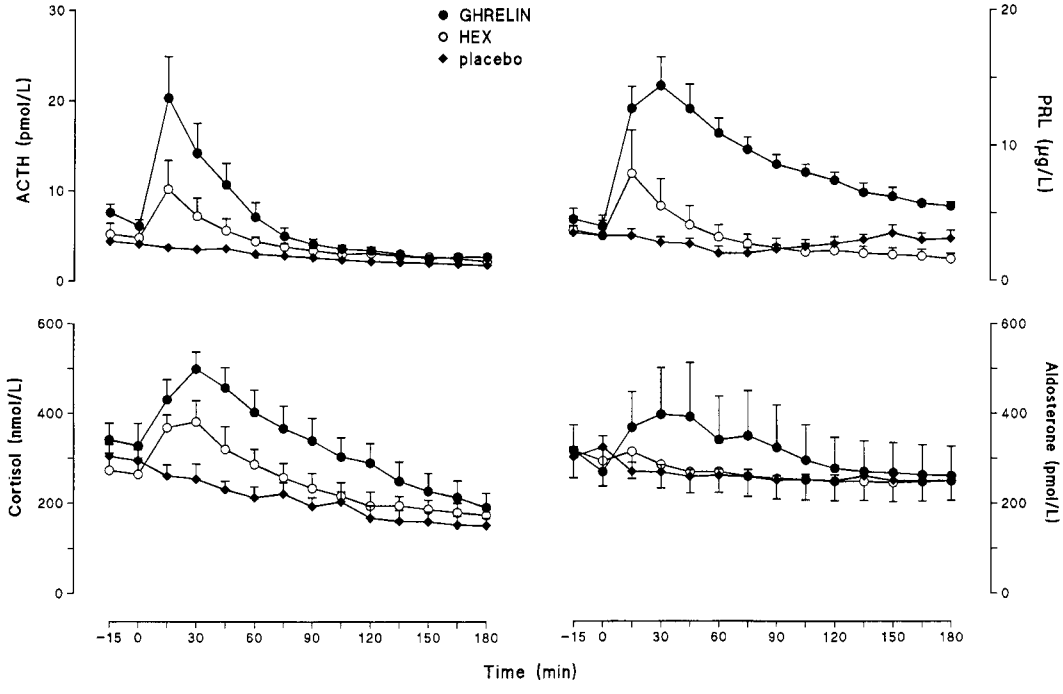

FIG. 2. Mean (± SEM) ACTH (*top left*), PRL (*top right*), cortisol (*bottom left*), and aldosterone (*bottom right*) curve responses after ghrelin (1.0 μg/kg), HEX (1.0 μg/kg), or placebo in normal subjects.

The endocrine responses to ghrelin were not modified by the coadministration with HEX. In fact, in six subjects, the GH response to ghrelin plus HEX was similar to that recorded after ghrelin alone (Cmax, 99.7 ± 12.9 *vs.* 100.3 ± 17.3 μg/L; AUC, 2265.9 ± 201.8 *vs.* 2066.0 ± 358.3 μg/L·h) (Fig. 3). Similarly, the PRL (Cmax, 15.9 ± 2.7 *vs.* 15.6 ± 2.4 μg/L; AUC, 570.7 ± 61.6 *vs.* 554.8 ± 49.7 μg/L·h), ACTH (Cmax, 24.2 ± 4.4 *vs.* 20.2 ± 5.0 pmol/L; AUC, 485.0 ± 87.3 *vs.* 407.2 ± 81.0 pmol/L·h), cortisol (Cmax, 620.4 ± 59.9 *vs.* 495.0 ± 43.4 nmol/L; AUC, 24537.4 ± 2575.6 *vs.* 20616 ± 2769.0 nmol/L·h), and aldosterone (Cmax, 534.7 ± 112.9 *vs.* 507.2 ± 106.9 pmol/L; AUC, 23958.9 ± 4501.3 *vs.* 22173.8 ± 4398.0 pmol/L·h) responses to ghrelin plus HEX overlapped with those after ghrelin alone.

On the other hand, the coadministration of ghrelin and GHRH had a synergistical effect on GH secretion. In fact, the GH response to ghrelin plus GHRH (Cmax, 133.6 ± 22.5 μg/L; AUC, 3374.3 ± 617.3 μg/L·h) was higher ($P < 0.05$) than the sum of the GH responses to ghrelin and GHRH alone (Fig. 3).

Side effects

No changes in heart rate and blood pressure were recorded after ghrelin administration, though three out of seven subjects were hungry at the end of the ghrelin testing session. Transient facial flushing was recorded in four out of seven subjects after GHRH administration and in two out of seven subjects after HEX administration. The coadministration of ghrelin and HEX or ghrelin and GHRH did not modify

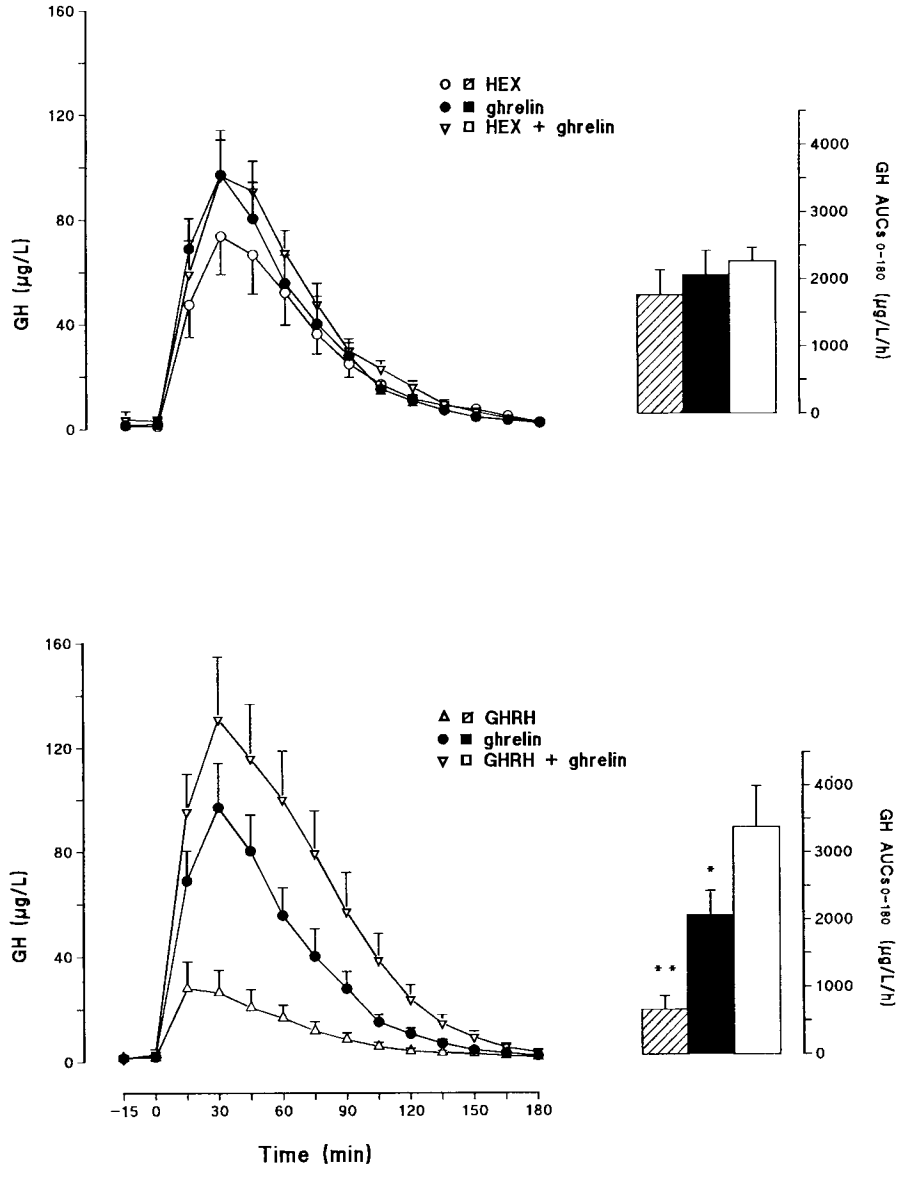

FIG. 3. Mean (± SEM) GH curve responses (*left*) and AUCs (*right*) after ghrelin (1.0 μg/kg), HEX (1.0 μg/kg), or ghrelin + HEX (*top*) and after ghrelin, GHRH (1.0 μg/kg), or ghrelin + GHRH (*bottom*) in normal subjects. *, $P < 0.05$ *vs.* ghrelin + GHRH; **, $P < 0.01$ *vs.* ghrelin + GHRH.

the side effects recorded after the administration of various peptides alone.

Discussion

The results of the present study show that in humans, ghrelin (a natural GHS-R ligand) possesses a strong stimulatory effect on GH secretion, releasing more GH than GHRH and even than a nonnatural GHS, such as HEX. The effect of ghrelin is, however, not fully specific for GH. In fact, ghrelin also stimulates lactotroph and corticotroph secretion and the ACTH response to ghrelin is, in turn, followed by an increase in cortisol and even in aldosterone levels. The endocrine responses to ghrelin are not modified by its coadministration with HEX; on the other hand, ghrelin has a synergistical effect, along with GHRH, on GH secretion.

It has been demonstrated that the strong GH-releasing activity of nonnatural GHS is mediated by actions on the pituitary and, mainly, on the hypothalamus, probably via the activation of GHRH-secreting neurons and/or via functional antagonism of somatostatin action (3, 4, 6, 14, 15). GHSs bind and activate both pituitaric and hypothalamic GHS-R (3, 8, 15–18), though receptor subtypes have been shown in the pituitary, in the brain, and in peripheral tissues (7, 12, 16, 18). Evidence that nonnatural GHSs act via specific orphan receptors suggested the existence of a natural GHS-like ligand (8). Both rat and human ghrelin have recently been isolated and proposed as an endogenous ligand of the GHS-R. In fact, this peptide is able to selectively stimulate GH secretion from both rat pituitary cells in culture and rat *in vivo* (9) and has recently been shown to inhibit the binding of ^{125}I-Tyr-Ala-hexarelin from pituitary and hypothalamic membranes in humans (12). More recently, another ligand, showing the same activity, has been purified from the rat stomach; it is a 27-amino-acid peptide named des-Gln14-ghrelin, whose sequence is identical to ghrelin except for one glutamine (10).

In a previous preliminary study, we showed that in four

normal young subjects, the administration of 1.0 μg/kg human ghrelin elicits a strong increase in circulating GH levels (13). The marked stimulatory effect of ghrelin on somatotroph secretion in humans is definitively demonstrated in the present study, in which it was compared with the one recorded after the administration of 1.0 μg/kg HEX hexapeptide, the effects of which had been extensively described in humans (4–6), or 1.0 μg/kg GHRH, the specific hypophysiotropic neurohormone (19). In agreement with evidence that nonnatural GHSs release more GH than GHRH (4, 6, 14), ghrelin (a 28-amino-acid peptide) released much more GH than GHRH-29, suggesting that ghrelin, a natural ligand of GHS-R, possesses a more potent GH-releasing activity than GHRH. It has also to be noted that ghrelin induced an even greater GH response than that elicited by the same absolute dose of HEX, known as one of the most potent peptidyl GHSs (4, 6). When considered on a molar basis, the present results suggest that ghrelin is more potent in releasing GH than synthetic nonnatural GHS.

This evidence is well suited to the hypothesis that ghrelin may be a true natural ligand of GHS-R (9, 15). This assumption is confirmed by evidence that human ghrelin does not show any interaction with HEX on GH secretion. The present findings in humans agree with preliminary results in rats showing that rat ghrelin and GHRP-2 have no interaction on somatotroph secretion (20). Interestingly, evidence that the GH response to ghrelin is not modified by the coadministration of HEX also indicates that the somatotroph responsiveness to ghrelin is reproducible; this property could have clinical implication whenever ghrelin is considered as a provocative test of GH secretion.

On the other hand, the evidence that human ghrelin and GHRH have a synergistical effect on GH secretion agrees with the well-known synergism between nonnatural GHS and GHRH (4, 6, 8). As previously hypothesized, based on the effect of nonnatural GHS, the synergistical interaction between ghrelin and GHRH indicates that these peptides act, at least partially, via different mechanisms.

In all, the present results emphasize the importance of ghrelin in the control of somatotroph function in humans and indicate that, actually, nonnatural GHSs were imitating its GH-releasing effect.

Our study also shows that the activity of ghrelin is not fully specific for GH release, at least in humans. In fact, different from what was recorded in the rat (9), the acute administration of human ghrelin induced increase in both lactotroph and corticotroph secretion.

Just as with nonnatural GHS, evidence that ghrelin also possesses PRL-, ACTH- and cortisol-releasing activity indicates that testing with nonnatural GHS really reflects the activities of the endogenous GHS-like ligand. Therefore, it is unlikely that the stimulatory effect of nonnatural GHS on lactotroph and corticotroph secretion could merely reflect loss of specificity of small synthetic molecules. In fact, in the present study, ghrelin and HEX had no interaction on both lactotroph and corticotroph secretion; and this evidence once again supports the hypothesis that ghrelin is a true natural ligand of GHS-R (9, 15).

The PRL-releasing activity of nonnatural GHS has been explained by direct action on the pituitary on somatomammotroph cells (21).

On the contrary, at least in physiological conditions, the ACTH-releasing effect of nonnatural GHS mainly depends on central nervous system-mediated mechanisms, possibly involving CRH- and/or AVP-mediated mechanisms (22, 23) but also partially independent of them (24). In fact, GABAergic pathways could play a major role in the GHS-induced ACTH release (25). Whether these mechanisms of action also explain the PRL- and ACTH-releasing activity of ghrelin is to be demonstrated.

In agreement with the results on GH secretion, the stimulatory effect of ghrelin on lactotroph and corticotroph secretion is more pronounced than nonnatural GHSs, such as HEX. The more marked ACTH-releasing activity of ghrelin is likely to explain the significant increase in aldosterone levels, which was not observed after HEX. In fact, it has been demonstrated that aldosterone levels are increased even by extremely low ACTH doses (26); moreover, the aldosterone response to some peptidyl GHSs endowed with marked ACTH-releasing effect has already been shown in humans (27). Based on the existence of specific GHS binding sites in the human adrenal gland (12), a direct action of ghrelin at this level cannot be ruled out.

In conclusion, the results of this study show that, in humans, ghrelin possesses very strong GH-releasing activity, beside a clear stimulatory effect also on lactotroph and corticotroph secretion. The endocrine responses to ghrelin are not modified by its coadministration with nonnatural GHS, whereas the GH-releasing effect of ghrelin and GHRH is synergistical. These findings indicate that the effects of nonnatural GHS were mimicking those of ghrelin, which is a new hormone playing a major role in the control of somatotroph function as well as other endocrine activities.

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