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Non-acylated ghrelin does not possess the pituitaric and pancreatic endocrine activity of acylated ghrelin in humans

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ABSTRACT. Ghrelin, a 28-amino acid peptide predominantly produced by the stomach, displays strong GH-releasing activity mediated by the GH secretagogue (GHS)-receptor (GHS-R) type 1a at the hypothalamus-pituitary level. Ghrelin and synthetic GHS also possess other GH-independent peripheral endocrine and non-endocrine activities via the activation of peripheral GHS-R subtypes. In rats *in vivo* non-acylated ghrelin has been reported devoid of any endocrine activity; however, *in vitro*, it has been shown as effective as ghrelin in exerting anti-proliferative activity on tumor cell lines. The aim of the present study was to clarify whether non-acylated human ghrelin shares some of the endocrine activities of its acylated form in humans. To this goal, the effects of acylated or non-acylated ghrelin (1.0 µg/kg iv at 0 min) on GH, PRL, ACTH, F, insulin and glucose levels were studied in two different testing sessions in 7 normal young volunteers (age [mean±SE]: 24.3±1.7 yr; BMI: 21.5±0.9 kg/m^2). The effects of placebo administration were also studied. The administration of acylated ghrelin induced prompt and marked increase in circulating GH levels (AUC: 5452.4± 904.9 µg*min/l; *p*<0.01 *vs* placebo) and significant increase in PRL (1273.5±199.7 µg*min/l; *p*<0.01 *vs* placebo), ACTH (4482.7±954.4 pg*min/ml; *p*<0.01 *vs* placebo) and F levels (15985.0±1141.9 µg*min/l; *p*<0.01 *vs* placebo). Its administration was also followed by decrease in insulin levels (1448.67±137.9 mU*min/l; *p*<0.05 *vs* placebo) that was coupled with an increase in plasma glucose levels (10974.2±852.5 mg*min/dl; *p*<0.05 *vs* placebo). The administration of non-acylated ghrelin and that of placebo did not induce any change in the hormonal parameters or in glucose levels. In conclusion, this study shows that in humans non-acylated ghrelin does not possess the pituitaric and pancreatic endocrine activities of human ghrelin octanoylated in Serine 3.

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INTRODUCTION

Ghrelin is a 28-amino acid peptide predominantly produced by the stomach, while substantially lower amounts derive from bowel, pancreas, kidneys, placenta, pituitary and hypothalamus (1-3). Ghrelin displays a strong GH-releasing activity mediated by the activation of the GH secretagogue (GHS)-Receptor (GHS-R) type 1a, which has been shown specific for the family of synthetic, peptidyl and non-peptidyl GHS (2, 4-6).

Key-words: Ghrelin, acylation, GH Secretagogues, GH, PRL, ACTH, insulin, glucose.

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GHS-R are concentrated in the hypothalamus-pituitary unit but also distributed in other central and peripheral tissues including the endocrine pancreas (5-9). Besides their potent GH-releasing effect, ghrelin as well as synthetic GHS has other remarkable GH-independent activities including: a) stimulation of lactotroph and corticotroph secretion (4-6, 10); b) orexigenia coupled with control of energy expenditure (3, 11); c) influence on sleep (5); d) control of gastric motility, acid secretion and exocrine pancreas secretion (2, 3, 12); e) influence on the endocrine pancreatic function and glucose metabolism (9, 13); f) cardiovascular actions including protection from ischemia and increase of the cardiac contractility *in vivo* (5, 14, 15); g) anti-proliferative effects in neoplastic thyroid, breast and lung cell lines (16-18).

Ghrelin circulates in acylated and non-acylated

form, this latter circulating in a higher amount (1, 2, 19). Based on studies in animals, it has been assumed that ghrelin is bioactive only after acylation in Serine 3 (1, 2, 20-22); note that this modification was observed for the first time in mammalian physiology (2). However, it has already been shown that the non-acylated form is as effective as acylated ghrelin in exerting some non-endocrine actions such as anti-proliferative activity on human breast carcinoma cell lines (17).

Based on the foregoing, we aimed to verify whether non-acylated ghrelin possesses endocrine activities in humans. To this goal, we compared the endocrine effects of the acute iv administration of acylated and non-acylated human ghrelin in normal young volunteers. Besides the effect on GH, PRL, ACTH and F secretion, we also studied the effects of both ghrelin forms on insulin secretion and glucose levels. In fact, acylated ghrelin has recently been shown able to induce hyperglycemia and to decrease insulin secretion in humans (13). Moreover, the expression of both ghrelin and GHS-R in the pancreas has already been shown (8, 9).

MATERIALS AND METHODS

Seven healthy young male volunteers [age (mean±SE): 24.3±1.7 yr; BMI: 21.5±0.9 kg/m^2] were studied. All subjects gave their written informed consent to participate in the study that had been approved by an independent Ethics Committee.

All subjects underwent the following three testing sessions in random order and at least 3 days apart: a) acylated ghrelin (1.0 µg/kg iv at 0 min; human octanoylated-ghrelin, vials 100 µg, Europeptides, Argenteuil, France); b) non-acylated ghrelin (1.0 µg/kg iv at 0 min; human des-octanoylated-ghrelin, vials 100 µg, Europeptides, Argenteuil, France); c) placebo (iv at 0 min).

After overnight fasting, the tests began in the morning at 08:30-09:00 h, 30 min after an indwelling catheter had been placed into a forearm vein kept patent by slow infusion of isotonic saline.

Blood samples were taken every 15 min from –15 min up to +120 min.

GH, PRL, ACTH, F, insulin and glucose levels were assayed at each time point in both sessions.

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 intra-assay coefficients of variation 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, Saluggia, Italy). The sensitivity of the assay was 0.15 µg/l. The inter- and intra-assay variation coefficients ranged between 3.9 and 6.8% and between 3.3 and 7.5%, respectively.

Plasma ACTH levels (pg/ml) were measured in duplicate by immunoradiometric assay (Allegro HS-ACTH, Nicholls Institute Diagnostic, San Juan Capistrano, USA). The sensitivity of the assay was 0.99 pg/ml. The inter- and intra-assay variation coefficients ranged between 6.9 and 8.9% and between 1.1 and 3.0%, respectively.

Serum F levels (µg/l) were measured in duplicate by radioimmunoassay (CORT-CTK 125, IRMA, SORIN, Saluggia, Italy). The sensitivity of the assay was 4.0 µg/l. The inter- and intra-assay variation coefficients ranged between 6.6 and 7.5% and between 3.8 and 6.6%, respectively.

Serum insulin levels (mU/l) were measured in duplicate by immunoradiometric assay (INSIK-5, SORIN Biomedica, Saluggia, Italy). The sensitivity of the 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 glucose levels (mg/dl) were measured by gluco-oxidase colorimetric method (GLUCOFIX, by Menarini Diagnostici, Florence, Italy).

All samples from an individual subject were analyzed together. The responses are expressed as absolute values or as AUC calculated by trapezoidal integration.

The statistical analysis was carried out using non-parametric ANOVA (Friedman test) and then Wilcoxon test, as appropriate. The results are expressed as mean±SE.

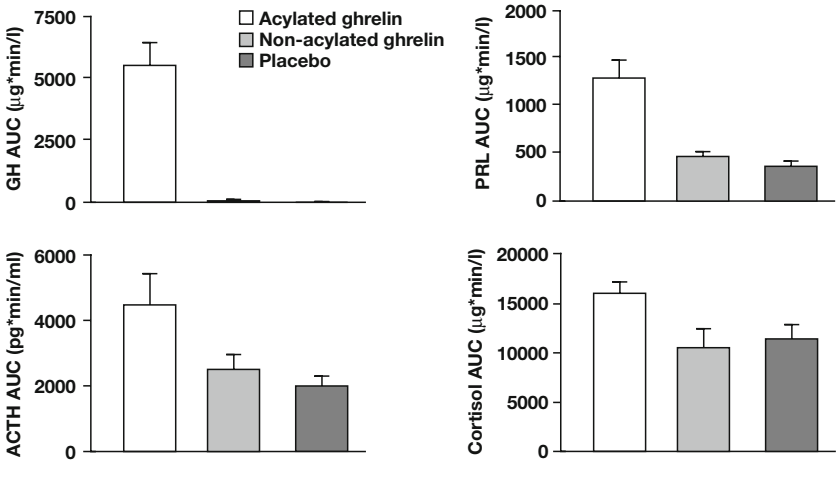

Fig. 1 - *Mean (±SE) GH, PRL, ACTH and cortisol responses to acute iv acylated ghrelin (1.0 µg/kg), non-acylated ghrelin (1.0 µg/kg) and placebo in normal subjects.*

RESULTS

The administration of acylated ghrelin induced a prompt and marked increase in circulating GH levels (AUC: 5452.4±904.9 µg*min/l; $p<0.01$ *vs* placebo). Acylated ghrelin induced also significant increase in PRL (1273.5±199.7 µg*min/l; $p<0.01$ *vs* placebo), ACTH (4482.7±954.4 pg*min/ml; $p<0.01$ *vs* placebo) and F levels (15985.0±1141.9 µg*min/l; $p<0.01$ *vs* placebo) (Fig. 1).

Administration of acylated ghrelin was also followed by decrease in insulin levels ($p<0.05$ *vs* placebo) starting at +30 min and showing its nadir at +45 min (10.3±0.9 mU/l, $p<0.01$ *vs* baseline). Insulin decrease was preceded by increase in plasma glucose levels ($p<0.01$ *vs* placebo) starting at +15 min and peaking at +75 min (95.7±9.7 mg/dl, $p<0.05$ *vs* baseline) (Fig. 2).

The administration of non-acylated ghrelin like that of placebo did not induce any change in hormonal parameters as well as in glucose levels. In fact, GH (55.9±48.9 *vs* 11.8±3.7 µg*min/l), PRL (462.6±45.7 *vs* 358.1±54.2 µg*min/l), ACTH (2501.4±464.5 *vs* 2002.7±289.4 pg*min/ml), F (10517.1±1902.9 *vs* 11332.9±1496.0 µg*min/l), insulin (1791.8±230.2 *vs* 1640.0±80.1 mU*min/l) and glucose (9080.4±102.7 *vs* 9986.3±297.1 mg*min/dl) curves and AUC after non-acylated ghrelin or placebo were overlapping (Fig. 1 and 2).

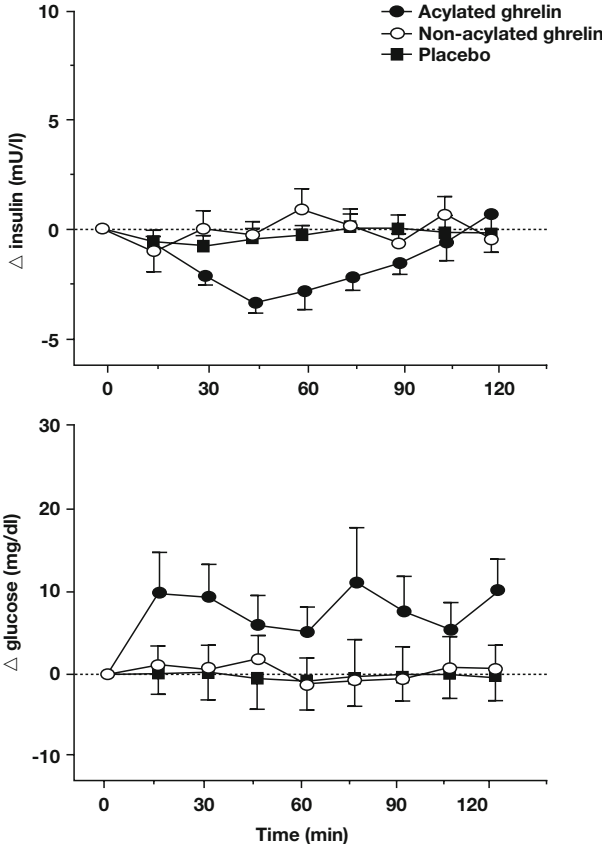

Fig. 2 - *Mean (±SE) △ insulin and glucose levels after acute iv acylated ghrelin (1.0 µg/kg at 0 min), non-acylated ghrelin (1.0 µg/kg at 0 min) and placebo in normal subjects.*

DISCUSSION

The results of the present study show that non-acylated ghrelin does not possess the pituitaric and pancreatic endocrine activities of human ghrelin octanoylated in Serine 3.

Ghrelin is a natural ligand of the GHS-R which had been shown specific for both peptidyl and non peptidyl, synthetic GHS possessing strong GH-releasing activity (1, 2, 4, 10, 23). Another natural ligand for the GHS Type 1a homologous to ghrelin except one glutamine missing has been isolated from the stomach and named des-Gln14-ghrelin; it is the result of an alternative splicing of ghrelin gene and possesses the same activity of ghrelin (2). Ghrelin and des-Gln14-ghrelin belong to a family of homologous gastro-entero-pancreatic hormones including also motilin and the recently discovered motilin-related peptide which however are not able to activate GHS-R and bind motilin-receptor which, in turn, shows high homology to GHS-R (3).

Ghrelin circulates in two forms simply differing on the acylation, the most abundant of which is non-acylated ghrelin (2, 24). Ghrelin is the first peptide isolated from natural sources in which the hydroxyl group of one of its serine residues is acylated by n-octanoic acid (2) and it is relevant that its biological activity has been assumed dependent on this (1, 2, 20-22). This assumption was based on evidence that non-acylated ghrelin was found unable to stimulate GH secretion in rats either *in vitro* or *in vivo* (1, 2). However, non-acylated ghrelin is not biologically inactive being able to share the same antiproliferative effect on human breast cancer cell lines with the acylated form (17). Moreover, acylated ghrelin was firstly reported to specifically stimulate GH without any effect on lactotroph and corticotroph secretion in rats (2) but then it was shown to also possess clear stimulatory effects on both PRL and ACTH secretion in humans (4, 10). Thus, we decided to verify whether non-acylated ghrelin were really devoid of any endocrine activity in humans. As ghrelin and GHS-R are expressed in the endocrine pancreas (8, 9) and acute ghrelin administration induces hyperglycemia and reduces insulin se-

cretion in young adults (13), the effect, if any, of non-acylated ghrelin was verified on these parameters as well.
Our findings confirm that acylated ghrelin strongly stimulates GH secretion but also significantly increases PRL, ACTH and F and induces hyperglycemia followed by insulin decrease (10, 13, 23). The GH-releasing effect of acylated ghrelin reflects action at the pituitary and, mainly, at the hypothalamic level where it acts through enhancing the activity of GHRH-secreting neurons and acting as a functional antagonist of somatostatin activity (5, 6). The slight PRL-releasing effect likely takes place directly at the pituitary level while the ACTH-releasing effect is totally dependent on central mechanisms probably including AVP-, NPY- and GABA-mediated actions (5). At present, the mechanisms underlying the effects of ghrelin on insulin and glucose levels are still unclear. However, evidence that the ghrelin-induced increase in glucose levels precedes the decrease in insulin levels, at least when measured peripherally, suggests that ghrelin would exert glycogenolitic activity in the liver (13). On the other hand, the inhibitory effect of ghrelin administration on insulin secretion could be mediated by a direct activity on the pancreatic β cells where specific GHS receptors have been shown (8, 9).
Actually, non-acylated ghrelin did not share any of the effects of the acylated ghrelin form thus definitely demonstrating that all the endocrine activities of ghrelin totally depend on the octanoylation in Serine 3.
The acylation of the peptide has been supposed critical to cross the blood-brain-barrier, however, it has also been demonstrated that acylation is also essential for binding the GHS Type 1a in the hypothalamus and the pituitary (21, 22, 25, Tschoep personal communication). It should be noted that non-acylated ghrelin is able to inhibit the binding of labeled ghrelin in human breast cancer cell lines (17); in these experimental conditions acylated and non-acylated ghrelin share the same antiproliferative effect thus showing they are probably acting on another GHS-R subtype (17).
In conclusion, this study in humans shows that non-acylated ghrelin is devoid of any of the endocrine activities displayed by this gastric hormone after octanoylation in its Serine 3. The biological impact of such remarkable quantity of circulating non-acylated ghrelin remains to be clarified but it seems clear from this study that it does not share with acylated ghrelin the critical task of driving the endocrine and metabolic responses to variations in the nutritional balance (3, 11, 26).

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