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Research paper

Ghrelin and growth hormone serum levels during the clonidine test in children with short stature and variable growth hormone status

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ABSTRACT

OBJECTIVE: The exact role of ghrelin in the control of growth hormone (GH) secretion has not been completely clarified as yet. The aim of the present study was 1) to investigate the effect of a substance promoting GH secretion (clonidine) on ghrelin levels in children with short stature with growth hormone deficiency (GHD) and normal growth hormone (NGH), and 2) to assess possible correlations between GH and ghrelin values during the clonidine test. DESIGN: Eighteen prepubertal children with short stature were included in the study. Using the results of two GH-provocative tests (glucagon and clonidine), the participants were divided into two groups: GHD and NGH. In both groups, ghrelin levels were determined during the clonidine stimulation test. RESULTS: Different responses regarding ghrelin levels during the clonidine stimulation test were observed in the two study groups (GHD and NGH). A decrease in ghrelin levels was observed in the NGH children accompanied by a rise in the circulating GH levels, whereas the GHD children demonstrated a rise in both ghrelin and GH levels. CONCLUSIONS: The data indicate an inverse relationship between circulating ghrelin and GH in NGH children, suggesting the presence of a negative feedback loop between ghrelin and GH. Analogous changes were not observed in GHD children.

Key words: Clonidine, Ghrelin, Growth, Growth hormone, Growth hormone deficiency, Short stature

INTRODUCTION

Ghrelin is a 28 amino-residue peptide produced mainly in the stomach.[1] Among its several endocrine activities, ghrelin also exerts orexigenic and adipogenic effects by modulating the expression of orexigenic and anorexigenic neuropeptides in the hypothalamus.[2-4] Ghrelin was initially identified as the endogenous ligand of the growth hormone secretagogue (GHS) receptor type 1a (GHS-R1a), possessing a strong growth hormone-releasing activity.[5,6] GHS receptors

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are present in the hypothamulus and the pituitary.[7,8] In addition, ghrelin potentiates the growth hormone releasing hormone (GHRH)-dependent secretion of growth hormone (GH).[9]

In general, the GH-releasing effect of ghrelin appears to result from several actions such as the ghrelin binding to GHSR-1a on the somatotrophic cells in the pituitary,[8] the ghrelin-induced activation of GHRH-containing neurons, the inhibition of somatostatin-containing neurons in the hypothalamus[10] and also the activation of vagal afferents.[11]

Nevertheless, the exact role of ghrelin in the control of GH secretion has not yet been clarified. Many studies have been conducted in short-stature children in order to assess the presence of a negative feedback loop between GH/IGF-1 axis and ghrelin using different GH provocative tests, with variable results.[12-15] The variability in the results might be attributed, at least in part, to the fact that ghrelin secretion is closely associated with glucose metabolism,[16] thus the use of insulin or glucagon for GH stimulation may be unsuitable. Clonidine, on the other hand, which has not thus far been used in studies assessing the relationship between ghrelin and GH, could represent a more appropriate stimulus, since it does not affect glucose metabolism.[17]

Therefore, the present study was designed to investigate: 1) the effect of clonidine on ghrelin levels in short-stature children with or without growth hormone deficiency and 2) to uncover possible correlations of circulation GH with ghrelin throughout the clonidine test.

PATIENTS AND METHODS

Patients

Eighteen children (11 boys and 7 girls), aged 9.7±2.6 (mean±SD) years, with short stature were studied. The participants were recruited from the outpatient unit of the 4th Department of Pediatrics, of the Aristotle University Medical School, Thessaloniki, Greece. Inclusion criteria were absence of chronic disease or intrauterine growth retardation, no drug therapy and an unremarkable family and personal medical history.

Body weight and height were measured in duplicate using a Seca weighing-scale (Seca 711) and a stadiometer (Harpenden, Baty International, Essex, UK), respectively. Standard deviation score (SDS) as well as body mass index (BMI) z-scores were calculated using the growth charts suggested by Kuczmarski et al.[18]

Physical examination was normal and pubertal stage was Tanner I (prepubertal).[19,20] The standard laboratory tests (full blood count, routine biochemical analysis including renal, liver and thyroid function) did not demonstrate any abnormality. Coeliac disease was excluded by normal values of anti-endomysium, anti-transglutaminase and anti-gliadin antibodies. Brain magnetic resonance imaging (MRI) was performed in all patients.

The study was approved by the Scientific Ethics Committee of the Aristotle University's Medical School. Informed written consent was obtained from parents and guardians of all participants prior to the investigation.

Procedure

Glucagon and clonidine stimulation tests (0.03 mg/kg of body weight IM, max 1 mg and 0.150 mg/m^2 orally, max 0.3 mg, respectively) were performed in all participants for GH assessment.[21,22] Ten of the children were diagnosed with growth hormone deficiency (GHD) using a peak GH cut-off value of 10 μg/l. Thus, the sample included two groups: the GHD (n=10) and the normal growth hormone (NGH) (n=8). Besides GH levels, ghrelin levels were additionally measured during the clonidine stimulation test.

At 08:30, following an overnight fast, an intravenous catheter was placed in the anticubital vein (half an hour prior to the study in order to avoid the stress from venepuncture). During the clonidine test blood samples were drawn at baseline and 30, 60, 90 and 120 min after ingestion of clonidine for GH and ghrelin measurement. Throughout the procedure blood pressure was strictly monitored. Blood samples were then centrifuged (10 min at 1500g) and the aliquots were stored at -20° C, until ghrelin was assayed according to the instructions suggested by Gröschl et al[23] IGF-1 levels were also measured at baseline.

All anthropometric and metabolic parameters of the study population are presented in Table 1.

Table 1. Anthropometric and metabolic parameters of the study population (mean±SD)

	GHD (n=10)	NGH (n=8)	P value
Boys/girls (n)	6/4	5/3	
Age (years)	9.9±0.7	9.5±1.1	0.758
Body weight (kg)	34.4±3.3	29.5±4.6	0.389
Height (cm)	124.7±6.5	125.5±4.5	0.377
Height SDS	-2.06±0.59	-1.42±0.77	0.071
BMI z-score	0.99±1.03	0.44±1.81	0.561
GH peak (μg/l)	4.6±3.5	14.2±2.4	0.001
Ghrelin 0 (pmol/l)	699.8±79.9	620.2±66.2	0.469
Ghrelin peak (pmol/l)	1224.9±539.0	680.2±112.1	0.013

BMI: Body mass index; GH: Growth hormone; IGF-1: Insulin-like growth factor-1; GHD: growth hormone deficiency; NGH: normal growth hormone.

Assays

GH

Serum GH was measured by immunoradiometric assay (IRMA) - DPC Immulite 2000, using reagents purchased from Diagnostics Products Corporation, Gwynned, UK, with Coefficients of Variation (CV) <7% and lower detection limits 0.01 μg/l.

IGF-1

Serum IGF-1 was measured by IRMA using reagents purchased from Diagnostic Systems Laboratories, Texas, USA. Sensitivity, intra-assay coefficients of variation (CV) and inter-assay CV were 0.03 μg/l, 4.5-7.1% and 4.8-8.8%, respectively.

Ghrelin

Serum ghrelin was measured by a commercial radioimmunoassay kit (Phoenix Pharmaceuticals, Inc. Belmont, CA, USA). This assay uses a ^{125}I-labeled ghrelin tracer and a rabbit polyclonal antibody against full-length, octanoylated human ghrelin that recognizes the acylated and des-acyl forms. The antiserum does not cross-react with any relevant peptide according to the information provided by the manufacturer. The lower and upper detection limits were 23.7 and 739.6 pmol/l, respectively. The intra- and interassay coefficients of variation (CV) were 5.30% and 13.61%, respectively.

Statistical analyses

All statistical analyses were performed with SPSS software, v. 15.0 (SPSS Inc, Chicago, Illinois). Normal distribution was assessed with the Kolmogorov-Smirnov test and statistical significance level was set at 5% (2-tailed). All data were normally distributed.

Differences between GHD and NGH children were assessed via the independent samples t-test. Changes in each time interval as compared with baseline during the clonidine stimulation test were evaluated with paired samples t-tests. Correlations were assessed by calculation of the Pearson's coefficient. Figures were plotted with Origin 7.0 SRO (Origin Lab Corporation, Northampton, MA, USA).

RESULTS

Baseline values and changes during the clonidine test

No differences were observed in age, BMI z-score, height SDS and ghrelin levels between the two groups. GH peak values were significantly higher and ghrelin peak values were lower in the NGH children compared to the GHD participants (p=0.001 and 0.013, respectively) (Table 1).

Subjects with GHD demonstrated a significant

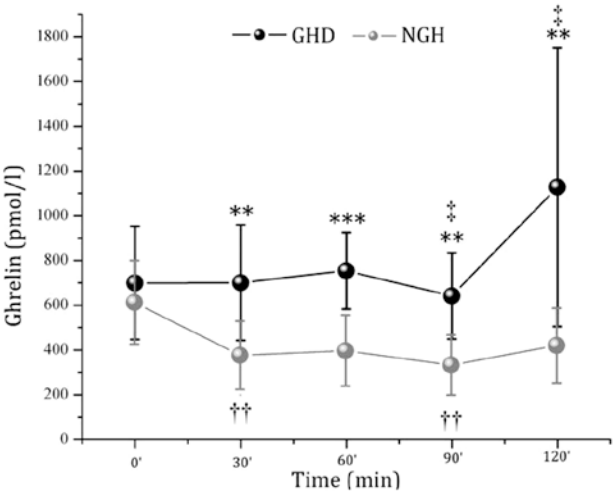

Figure 1. Mean ±SD values of serum ghrelin during the clonidine test in the two groups. Significantly different from baseline using paired t-test (†† p=0.01, † p=0.05). Significantly different from the previous time interval using paired t-test (‡ p=0.05). Significantly different from the NGH group using independent t-test (** p=0.01; *** p=0.001).

fall in serum ghrelin concentration at 90 min and a rise at 120 min (p=0.02 for both) compared to the previous time interval (Figure 1). In the NGH group, circulating ghrelin was significantly lower at 30 min and 90 min compared to baseline (p=0.009 and 0.007, respectively). Between the two studied groups, ghrelin levels were significantly higher in the GHD group at all time intervals with a p value of 0.007 at 30 min, 0.001 at 60 min, 0.002 at 90 min and 0.007 at 120 min post-clonidine administration. Individual and group fluctuations in circulating ghrelin are presented in Table 2.

Mean GH and ghrelin fluctuations within each group are demonstrated in Figure 2. GH concentration was higher in the NGH participants at 60 min and 90 min (p=0.011 and p=0.001, respectively) compared to the GHD children. GH levels significantly rose at 90 min (p=0.003) in the GHD participants and at all time intervals after 60 min in the NGH participants, compared to baseline (p=0.009, 0.003 and 0.041 at 60, 90 and 120 min, respectively).

Correlations

The absolute body weight (kg) was negatively correlated with the baseline ghrelin levels (r=-0.867, p<0.001), and to the baseline GH concentration (r=-0.787, p=0.018) only in NGH participants. In the NGH group, IGF-1 demonstrated a strong correlation with the height-SDS (r=0.843, p=0.009). The same group exhibited a negative relationship between baseline ghrelin and GH (r=-0.767, p=0.026). No correlations were observed between ghrelin and GH changes at the same time point. Ghrelin levels at 60min were significantly negatively correlated with GH levels at 90 min in the NGH participants (r=-0.735, p=0.001), indicating that a decline in ghrelin concentration precedes the increases in GH in this group.

DISCUSSION

The study population included short prepubertal children (with and without GHD). The selected prepubertal stage was chosen in order to avoid possible

Table 2. Individual and group ghrelin levels (pmol/l)

	Time after administration of clonidine (min)				
Participant	**0**	**30**	**60**	**90**	**120**
GHD #1	1227.3	1151.3	677.4	737.8	778.4
GHD #2	589.3	690.0	618.2	465.4	409.8
GHD #3	524.7	234.9	382.0	182.7	284.7
GHD #4	583.5	991.7	956.1	811.4	1843.4
GHD #5	740.2	607.4	903.9	759.2	1271.6
GHD #6	747.3	602.6	685.6	785.3	1276.4
GHD #7	310.8	491.1	899.2	605.0	2351.1
GHD #8	801.9	707.0	830.4	614.5	875.4
GHD #9	546.9	876.5	765.3	689.7	978.7
GHD #10	926.2	656.0	821.6	765.5	1210.7
NGH #1	813.2	305.6	252.4	504.8	335.8
NGH #2	218.6	215.3	537.2	261.7	400.6
NGH #3	827.1	569.6	407.5	252.4	289.4
NGH #4	664.7	204.0	185.1	130.5	244.4
NGH #5	618.9	624.0	664.3	540.9	780.5
NGH #6	625.5	378.5	407.3	336.4	411.3
NGH #7	573. 5	428.3	489.6	365. 5	520.7
NGH #8	620.1	345.4	287.5	330.3	435.6
GHD (mean±SD)	699.8±252.7	700.9±258.2**	754.0±170.7***	641.6±192.6‡**	1128.0±623.0‡**
NGH (mean±SD)	620.2±187.3	383.8±152.4††	403.8±159.2	340.3±134.2††	427.3±167.1

GHD: growth hormone deficiency; NGH: normal growth hormone.
Significantly different from baseline using paired t-test † p=0.05 and †† p=0.01. Significantly different from the previous time interval using paired t-test (‡ p=0.05). Significantly different from the NGH group using independent t-test (** p=0.01; *** p=0.001).

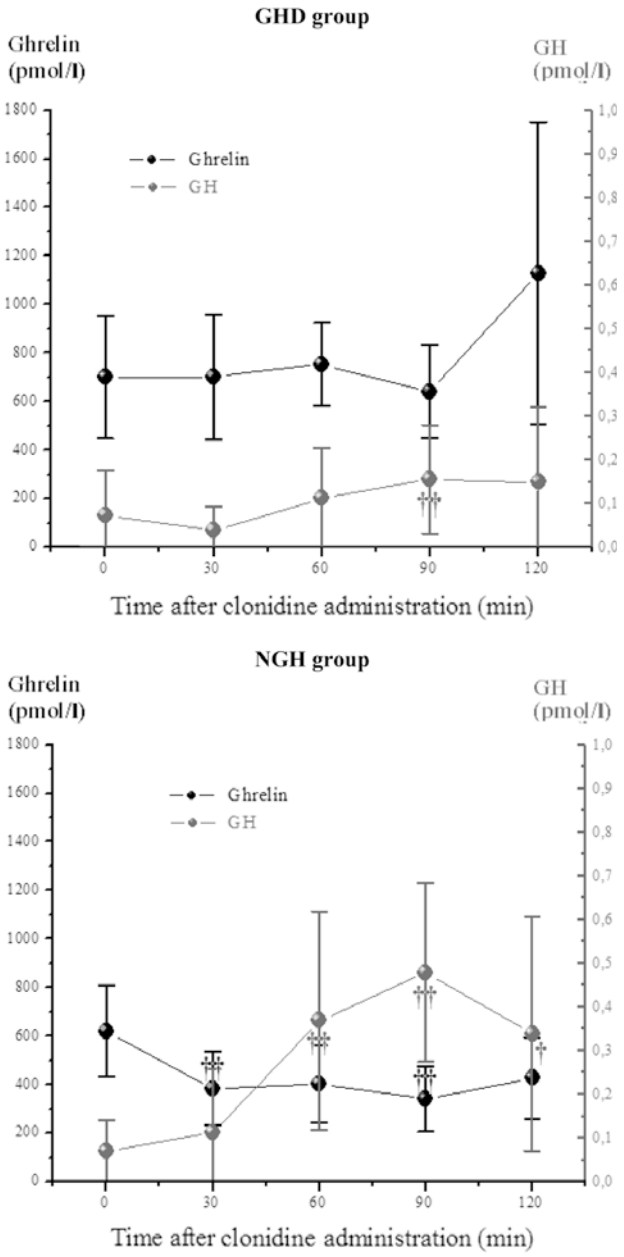

Figure 2. Serum ghrelin and GH values (mean±SD) during the clonidine test in the two groups. Significantly different from baseline using paired t-test (†† p=0.01, † p=0.05).
To convert GH values from pmol/l to μg/l, divide by 0.0465.

influence of puberty, which has been found to affect ghrelin levels.[24] According to our findings, clonidine ingestion induced different trends in the ghrelin levels in the GHD and NGH groups. Overall, ghrelin levels were higher in the GHD group compared to the NGH participants at all intervals after clonidine intake, but not at baseline. This observation is difficult to interpret but it may represent a compensatory mechanism for the low GH values through a negative GH-ghrelin loop effect.

There is scant information regarding the participation of ghrelin in the control of GH secretion. Although pituitary ghrelin appears to be involved in the GH release,[25] studies in patients with short stature of various etiology, using different GH provocative tests, have demonstrated conflicting results regarding the presence of a negative feedback between GH and ghrelin.[12-15] Two studies[12,26] have suggested an inverse relationship between circulating GH and ghrelin levels, indicating the existence of a feedback loop. Hirsh et al failed to relate ghrelin levels to the post-glucagon administration GH release, a finding explained by the authors as a result of the suppressed ghrelin exhibited after glucagon administration.[15] According to Janssen et al, GH replacement therapy for one year failed to alter the circulating ghrelin levels in subjects with GHD.[14] However, long-term treatment with recombinant human GH was found to be associated with a significant reduction in the BMI and %BF in GHD patients, and hence the attenuated ghrelin levels might be the result of altered body fat stores.[26]

The present study demonstrated different ghrelin-GH responses in the two study groups. In accordance with Matsuoka et al,[12] an inverse association was noted between circulating GH and ghrelin levels in the NGH participants, indicating the presence of a negative feedback loop between GH and ghrelin. Moreover, NGH participants exhibited a decline in total ghrelin concentration prior to the GH peak. Thus, an inverse relationship between ghrelin at 60 min and GH at 90 min was detected in the NGH group. This finding indicates that in NGH children, the rise in GH levels is associated with a decline in the circulating ghrelin values. It is possible that a feedback phenomenon occurs, ghrelin being suppressed by the rise in GH provoked by clonidine, although other mechanisms may be operative.

According to Maghnie et al,[27] children and adults with GHD demonstrate a rise in GH levels following an increase in the circulating ghrelin. The same investigators, using brain MRI, also demonstrated that GH response to ghrelin requires the functional

and anatomical integrity of hypothalamic-pituitary connections (intact pituitary stalk) and that the degree of the GH response to the releasing peptide might predict the degree of functional impairment of hypothalamic-pituitary connections. In our study population, brain MRI did not reveal any hypothalamic-pituitary abnormalities.

The negative relationship between baseline ghrelin and body weight observed in the NGH participants has also been noted by other researchers in children with idiopathic short stature.[28,29] This phenomenon has been explained as ghrelin's compensatory mechanism to change the metabolic process.[29,30] Since this relationship was only observed in NGH children, it is possible that the reduced GH levels in the GHD participants prohibits physiological ghrelin secretion and results in a different relationship between ghrelin and body weight in this group.

Certain limitations of the present study must be mentioned. First of all, the number of patients included was relatively small. Secondly, we did not perform a clonidine stimulation test on a group of prepubertal children with normal stature. This was not carried out for ethical reasons. Thirdly, the duration of the oral clonidine test is not standardized, the duration varying from 90-180min in different studies.[31] This inconsistency might explain the variable results obtained in different studies.

IGF-1 has been reported to be one of the most significant determinants of ghrelin secretion during childhood and adolescence,[32] as serum ghrelin and IGF-1 concentrations are negatively correlated in healthy children[33] and children with idiopathic short stature.[34] However, our study failed to demonstrate the existence of a relationship between ghrelin and IGF-1, possibly due to the small number of participants involved.

In conclusion, our findings indicate an inverse relationship between ghrelin and GH in NGH children suggesting the existence of a GH-ghrelin feedback loop. Analogous interrelationship was not observed in the GHD children, indicating that the GH status possibly determines the relationship between circulating levels of ghrelin and GH. Further studies are required to better define the relationship between ghrelin and GH and the underlying mechanisms.

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