CLINICAL STUDY

Partial restoration of GH responsiveness to ghrelin in Cushing's disease after 6 months of ketoconazole treatment: comparison with GHRP-6 and GHRH

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Abstract

Objective: In Cushing's disease (CD), GH responsiveness to several stimuli, including ghrelin, GHRP-6, and GHRH, is blunted. Recovery of GH secretion after remission of hypercortisolism after transsphenoidal surgery, radiotherapy, or adrenalectomy is controversial. There are no studies evaluating the effect of primary clinical treatment with ketoconazole on GH secretion in CD. The aim of this study is to compare ghrelin-, GHRP-6-, and GHRH-induced GH release before and after ketoconazole in CD.

Design: GH responses to ghrelin, GHRP-6, and GHRH of eight untreated patients with CD (mean age: 33.8 ± 3.1 years; body mass index: 28.5 ± 0.8 kg/m^2) were evaluated before and after 3 and 6 months of ketoconazole treatment, and compared with 11 controls (32.1 ± 2.5; 25.0 ± 0.8).

Methods: Serum GH was measured by an immunofluorometric assay and urinary free cortisol (UFC) by liquid chromatography and tandem mass spectrometry.

Results: After ketoconazole use, mean UFC decreased significantly (before: 222.4 ± 35.0 µg/24 h; third month: 61.6 ± 10.1; sixth month: 39.1 ± 10.9). Ghrelin-induced GH secretion increased significantly after 6 months (peak before: 6.8 ± 2.3 µg/l; sixth month: 16.0 ± 3.6), but remained lower than that of controls (54.1 ± 11.2). GH release after GHRP-6 increased, although not significantly, while GH responsiveness to GHRH was unchanged.

Conclusions: Ghrelin-induced GH release increases significantly after 6 months of ketoconazole treatment in CD. This could suggest that a decrease in cortisol levels during this time period can partially restore glucocorticoid-induced GH suppression in CD. GH-releasing mechanisms stimulated by ghrelin/GHS could be more sensitive, as no changes in GHRH-induced GH release were observed.

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Introduction

GH secretion is modulated by GHRH and somatostatin (SRIF), but GH secretagogues (GHS) might also have a role, acting at both hypothalamic and pituitary receptors (1, 2). Ghrelin, the endogenous ligand of GHS receptors (GHS-R), is present in the stomach and in the hypothalamus (3). The active acylated peptide has a different chemical structure than GHS (4). Ghrelin and GHRP-6, a GHS, are able to induce GH, ACTH, and cortisol release (5). Their main site of action is the hypothalamus, as these effects are reduced/abolished in hypothalamic–pituitary disconnection (6, 7). Ghrelin/GHS action on GH secretion probably involves activation of GHRH neurons, with increased GHRH release, amplification of GHRH effects at the somatotroph and functional antagonism of SRIF (8, 9). GHS/ghrelin and GHRH bind to separate receptors and activate different intracellular mechanisms at the somatotroph. GHRH stimulates cyclic AMP and protein kinase A pathways (3), while ghrelin and GHRP-6 activate protein kinase C signal transduction (2, 3). Moreover, there is a synergistic effect of ghrelin/GHS and GHRH on GH release (4).

In patients with Cushing's disease (CD), GH responsiveness to several stimuli, including ghrelin, GHRP-6, and GHRH, is blunted (10–12). The mechanisms involved in the inhibitory effect of chronic hypercortisolism on GH secretion are not clear. An increase in hypothalamic SRIF release is unlikely to have a major role (11). A decrease in GHRH secretion (13) and a direct effect of glucocorticoids at the somatotroph (14) might be involved.

It has been shown that GH secretion ability can be acutely enhanced in CD (14, 15). However, there are several reports of lack of recovery of GH secretion after

remission of hypercortisolism with transsphenoidal surgery (TS) or radiotherapy (RT) (16–22), which could be due, at least in part, to treatment-induced somatotroph damage. In contrast, early normalization of GH secretion has been observed when adrenalectomy is performed, and the pituitary is intact (16).

Ketoconazole is a steroidogenesis inhibitor that has been largely used for the treatment of Cushing's syndrome, as it is able to normalize cortisol levels in 70% of these patients (23) and rarely causes hepatic damage (24).

There are no data in the literature about the effect of clinical treatment with ketoconazole on GH secretion in patients with CD.

Therefore, the aim of this study was to evaluate GH responses to ghrelin and GHRP-6 before and after 3 and 6 months of ketoconazole use in untreated patients with CD. GHRH-induced GH release was additionally studied, as it stimulates GH secretion via different mechanisms than those of ghrelin/GHRP-6.

Material and methods

Subjects

Eight female patients with CD were studied before any treatment. Six had microadenomas and two had small macroadenomas (maximal tumor diameters of 11 and 13 mm). Their mean age ($\pm$ S.E.M.) was 33.8 ± 3.1 years (range: 19–41) and their body mass index (BMI) was 28.5 ± 0.8 kg/m^2 (range: 26.2–32.1). Diagnosis of CD was established by clinical features and standard hormonal criteria, including increased free urinary cortisol excretion, lack of suppression of serum cortisol after dexamethasone (1 mg orally overnight), normal or high basal plasma ACTH and serum cortisol levels at 0800 h, and positive desmopressin (DDAVP) test. Magnetic resonance imaging showed a pituitary adenoma in all patients. None of the patients was on replacement therapy for hypopituitarism before surgery. At diagnosis, all patients had normal renal function and four had secondary diabetes mellitus. One patient was treated with diet, two with metformin, and one with metformin and insulin.

After the end of the experimental protocol, all patients were submitted to TS and the diagnosis of ACTH-secreting tumor was confirmed by positive ACTH immunostaining of the excised pituitary adenoma.

Eleven normal subjects (four women and seven men) with a mean age of 32.1 ± 2.5 years (range: 20–47) and BMI of 25.0 ± 0.8 kg/m^2 (range: 20.9–30.8) were also studied. They were free of any medication during the study protocol. The women were tested in the early follicular phase of their menstrual cycle.

Study protocol

The experimental protocol was approved by the Ethics Committee of UNIFESP, and all subjects gave prior informed consent.

Each subject was submitted to three tests, in random order, with a minimum interval of 48 h, with acylated ghrelin (Neosystem, Strasbourg, France), GHRP-6 (Bachem, San Carlos, CA, USA), or GHRH (1–29) NH2 (Clinalfa, Läufelfingen, Switzerland). Patients with CD were studied before and after 3 and 6 months of ketoconazole use, which was given at a dose of 400 mg/day initially, administered two or three times a day. Urinary free cortisol (UFC) was measured every month, and the dose of ketoconazole was titrated accordingly (400–1200 mg/day).

All tests were performed after an overnight fast, and the subjects remained recumbent throughout. Patients were advised to take ketoconazole before the tests at the same time as usual (0600 h). At 0800 h, an indwelling catheter was inserted into an antecubital vein and was kept patent by a saline infusion. The tests started 45 min later. After the first blood sample, each subject received ghrelin or GHRP-6 at a dose of 1 µg/kg i.v. or GHRH at a dose of 100 µg i.v. Blood samples were obtained every 15 min until 120 min for hormonal and biochemical determinations.

Assays

Serum GH was measured in duplicate by an immunofluorometric assay (Wallac, Turku, Finland). The sensitivity of the method is 0.01 µg/l, with mean inter- and intraassay coefficients of variation (CV) of 7 and 6.7% respectively. Serum cortisol levels were measured in duplicate by a fluoroimmunoassay (Wallac), with sensitivity of 0.2 µg/dl, and mean inter- and intraassay CV of 8.2 and 6.2% respectively. Insulin-like growth factor-1 (IGF1) levels were determined by an immunochemiluminometric assay (DPC, Los Angeles, CA, USA), with sensivity of 20 ng/ml, and mean inter- and intraassay CV of 6.5 and 3.8% respectively. UFC was measured by liquid chromatography and tandem mass spectrometry (Fleury Laboratory, São Paulo, Brazil) (25), with sensitivity of <1 µg/l, and mean inter- and intraassay CV of 7.7 and 4.4% respectively.

Statistical analysis

Repeated-measures ANOVA or Friedman's ANOVA was used to compare data within the same group when appropriate. Unpaired *t*-test or Mann–Whitney rank sum test was performed to compare data between patients and controls. The area under the curve (AUC) was calculated by trapezoidal integration. Delta (Δ) values subtracting baseline or during treatment were also calculated. Spearman's correlation coefficient was

calculated when appropriate. Undetectable GH (µg/l) values were considered to be equal to 0.01 for statistical purposes. $P<0.05$ was considered statistically significant. Results are reported as mean±S.E.M.

Results

Basal values and clinical data

There was no difference in age between patients and controls. Before treatment, patients with CD had significantly higher BMI values compared with the control group. There was a trend to a decrease in mean BMI during treatment ($P=0.08$), and after 6 months, BMI values were similar to those of controls. Basal GH and IGF1 levels in CD were not different than those of controls initially and did not change significantly during treatment. At diagnosis, patients with CD had higher basal serum cortisol levels compared with controls. These values decreased significantly during ketoconazole use, but were still higher than those of controls at the third and sixth month. In CD, mean fasting glucose levels were higher than those of controls and decreased significantly during treatment, becoming similar to the control group at the sixth month (Table 1). All diabetic patients improved their glycemic control with ketoconazole treatment, with normalization of glucose levels in one patient and insulin withdrawal in another.

UFC values

Patients with CD had mean UFC (µg/24 h) of 222.4 ±34.5 at the time of the diagnosis. After 3 and 6 months of ketoconazole treatment, mean UFC decreased significantly to 61.6 ± 10.1 (mean reduction: 70%) and 39.1 ± 10.9 (mean reduction: 80%) respectively (Table 1). All patients showed a decrease in UFC values after 3 months of treatment, and after 6 months four patients had their values within the normal range, while two had UFC levels near the upper limit of normality. Only one patient did not show normalization of UFC values throughout the whole period, despite a major decrease both at the third (74%) and sixth (76%)

month. Another patient had an 'escape' at the sixth month, after normalization in the third month. None of the patients had UFC values below the normal range throughout the treatment (Table 1).

GH responses

Patients with CD had significantly lower peak GH (µg/l) and AUC (µg/l 120 min) values after ghrelin (6.8 ± 2.3; 238 ± 88), GHRP-6 (2.8 ± 0.8; 107 ± 37), and GHRH (1.1 ± 0.2; 64 ± 14) compared with the control group (ghrelin: 54.1 ± 11.2; 3123 ± 707; GHRP-6: 25.7 ± 4.5; 1396 ± 284; GHRH: 11.7 ± 3.3; 857 ± 267). After 3 months of ketoconazole use, GH responses to ghrelin (peak: 7.9 ± 2.2; AUC: 273 ± 83), GHRP-6 (peak: 4.1 ± 1.0; AUC: 149 ± 30), and GHRH (peak: 1.2 ± 0.3; AUC: 73 ± 18) did not change significantly (Figs 1 and 2). After 6 months, there was a significant increase in GH response to ghrelin in CD (peak: 16.0 ± 3.6; AUC: 602 ± 175) compared with before treatment and also with the third month of ketoconazole use. However, GH responsiveness to ghrelin in CD remained lower than in controls. GH response to GHRP-6 (peak: 5.1 ± 1.5; AUC: 194 ± 71) also increased, but did not reach statistical significance (Fig. 1). Individual analysis showed that all patients, except one, had an increase in GH responsiveness to ghrelin, and this was also seen in the majority after GHRP-6 stimulation. In three patients, peak GH values were within the range observed in the control group, while in one patient GH levels were just below the lower limit of controls for both ghrelin and GHRP-6. The only patient who failed to increase GH values after ghrelin had lack of normalization of UFC levels during the whole study period. When this patient was excluded from the statistical analysis, no significant differences were observed. GHRH-induced GH release (1.2 ± 0.4; 69 ± 21) did not change after 6 months of treatment, and only two patients reached values just above the lower limit of controls.

No correlations were found between Δ BMI, Δ UFC, Δ basal cortisol or Δ fasting glucose, and Δ GH peak and AUC after 6 months of treatment. Negative correlations

Table 1 Basal values and clinical data of patients with Cushing's disease (CD; $n=8$) before and after 3 and 6 months of ketoconazole treatment and of controls ($n=11$).

		CD		
	Controls	Before	Third month	Sixth month
Age (years)	32.1±2.5	33.8±3.1		
BMI (kg/m²)	25.0±0.8	28.5±0.8*	27.6±0.7*	27.2±0.8
GH (µg/l)	0.1±0.07	0.3±0.1	0.2±0.03	0.3±0.05
IGF1 (ng/ml)	177.2±12.6	232.4±41.6	225.5±23.1	220.5±30.4
UFC (µg/24 h)		222.4±34.5	61.6±10.1[†]	39.1±10.9[†]
Cortisol (µg/dl)	7.9±0.7	22.2±2.5*	16.8±1.6*,[†]	14.6±1.6*,[†]
Glucose (mg/dl)	84.6±2.2	113.1±10.4*	101.0±6.1*,[†]	92.8±5.8[†]

*$P<0.05$ versus controls; [†]$P<0.05$ versus before treatment.
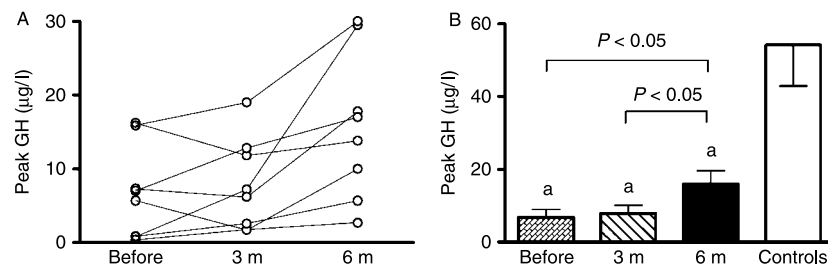
Figure 1 Individual (A) and mean GH values (B) after ghrelin administration in CD ($n=8$) before and after 3 and 6 months of ketoconazole treatment and in controls ($n=11$; mean$\pm$S.E.M.; a, $P<0.05$ versus controls).

between basal serum cortisol levels and GH AUC values after all peptides (ghrelin: $r=-0.762$, $P=0.02$; GHRP-6: $r=-0.762$, $P=0.02$; GHRH: $r=-0.755$, $P=0.02$) were observed after 6 months of treatment.

Side effects

Hunger sensation, nausea, and sleepiness were reported occasionally after ghrelin administration. Three patients had a transient and mild increase in alanine transaminase after the first month of ketoconazole administration.

Discussion

In our present study, patients with CD had blunted GH responses to ghrelin, GHRP-6, and GHRH before ketoconazole treatment, as previously reported by us and others (10–12). GH responsiveness to ghrelin was higher both in CD and controls, which could be due to the greater potency of this peptide (5).

It has been shown that adult patients with CD previously submitted to surgery and/or RT (16–22) have lack of recovery of GH secretion, which could be due, at least in part, to GH deficiency caused by pituitary damage. We, therefore, evaluated GH secretion in CD after pharmacological reduction or correction of hypercortisolism in patients who are not submitted to any pituitary intervention. Moreover, as most of our patients had microadenomas, with only two harboring small macroadenomas, they were likely to have an intact GH axis. It has been previously shown that acute clinical interventions such as inhibition of free fatty acids and short-term dietary restriction (14, 15) were able to increase GH secretion in CD. However, we did not observe significant changes in GH responses to ghrelin, GHRP-6, and GHRH after a short period (1 month) of ketoconazole use in patients with CD, although UFC values had a considerable fall (26).

Despite major decreases in UFC levels since the third month, GH responsiveness to ghrelin only increased significantly after 6 months of ketoconazole treatment. However, it remained lower than that seen in the control group. GHRP-6-induced GH release also increased, but not significantly, while no changes in

GH responses to GHRH were observed. Individual analysis showed that 50% of our patients had peak GH values after ghrelin/GHRP-6 within or near the range of controls after 6 months of treatment, while only two reached this range after GHRH stimulation. At this time point, six patients had normal or near normal UFC values. The only patient who did not increase GH values after ghrelin had UFC values above the upper limit of normality during the entire study period. No correlation was observed between UFC or serum cortisol levels and GH responsiveness to ghrelin, which has already been shown with other stimuli (21, 22). Periods of subtle hypo- or hypercortisolism, due to the pharmacological profile of ketoconazole, cannot be totally excluded and could have contributed to the partial improvement of the somatotrophic axis in some of our patients. However, our results are similar to those obtained by Tzanela *et al.* who showed recovery of GH secretion after
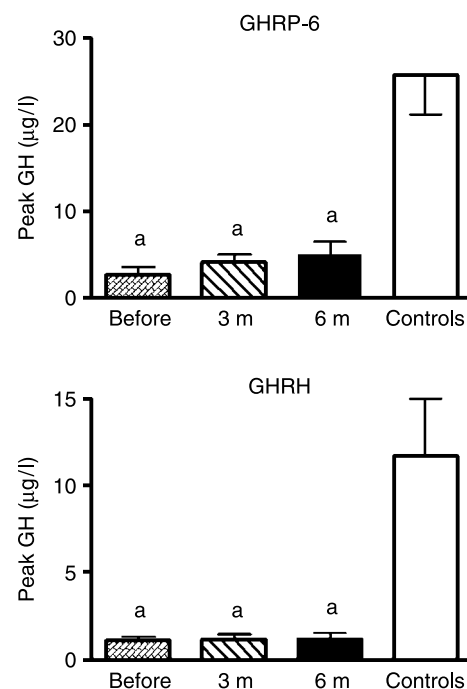
Figure 2 Mean GH values after GHRP-6 and GHRH administration in CD ($n=8$) before and after 3 and 6 months of treatment and in controls ($n=11$; mean$\pm$S.E.M.; a, $P<0.05$ versus controls).

pyridostigmine + GHRH and/or insulin tolerance test (ITT) in 50% (two out of four) of patients with CD, who had intact pituitary function, and who were in remission 6 months after TS (21). In contrast, lower GH recovery rates have been described in the first 6 months after TS in most studies (17–19). Our results suggest that, if there is no pituitary damage, even relatively short-term periods of normal or near normal cortisol values are able to partially restore GH secretion in CD. Moreover, this is apparently unrelated to a decrease in circulating IGF1, as no significant changes in IGF1 levels were observed in our study period, confirming previous data in patients with CD after TS (19, 21).

Obesity and hyperglycemia, two features of hypercortisolism, are associated with blunted GH responses to ghrelin, GHRP-6, and GHRH (27, 28) and could be contributing factors to the partial recovery of GH axis in some cases. However, we have previously shown that blunted GH responses also occur in overweight patients with CD compared with BMI-matched controls (12). Moreover, although our patients had slightly higher BMI values initially, these values became similar to controls after 6 months of ketoconazole use, and no correlation was found between GH responses to ghrelin and BMI in CD, as previously reported (12, 21, 22). Despite a significant decrease in fasting glucose levels during treatment, no correlation was found between glucose values and GH responsiveness to ghrelin. Therefore, the partial recovery of GH axis in some of our patients is unlikely to be due to these factors.

The possible mechanisms of decreased GH secretion and also of recovery of somatotroph responsiveness in CD remain unknown.

It has been previously suggested that glucocorticoid excess inhibits hypothalamic GHRH release (13), which could lead to chronic GHRH deficiency. In this situation, there is a lack of GH responsiveness to exogenous GHRH (29). Moreover, GHRH-mediated pathways at the somatotroph could also be impaired by hypercortisolism (14). Although controversial in animals (30, 31), this steroid also downregulates human GHS-R (32). Therefore, glucocorticoids could eventually interfere with ghrelin/GHS-stimulated transduction mechanisms at the somatotroph, with a possible additional effect on GHS-R located in GHRH-releasing neurons at the hypothalamus (9). As GHS/ghrelin and GHRH activate different pathways of GH release, the enhancement of ghrelin/GHRP-6-induced GH release after treatment together with lack of changes in GH responsiveness to GHRH could eventually suggest that ghrelin/GHS-modulated mechanisms are more sensitive to the decrease in circulating glucocorticoid levels than those of GHRH. Moreover, this is more pronounced for ghrelin than for GHRP-6, which could be due to the activation of multiple intracellular mechanisms in the somatotroph by ghrelin (33).

It is well established that there is a synergistic effect of ghrelin/GHS and GHRH on GH release (4). It has been recently shown that GHRH acts as a coagonist of GHS-R and increases the affinity of ghrelin by its receptor (34). Our data suggest that chronic GHRH deficiency may persist after 6 months of ketoconazole treatment, as no changes in GHRH-induced GH release were observed. This could interfere with the synergistic effect between ghrelin/GHS and GHRH, and eventually explain why GH responsiveness to ghrelin is not fully restored.

However, additional studies are necessary to confirm these hypotheses.

In summary, our results show that mean GH responsiveness to ghrelin increases, although does not normalize, after 6 months of ketoconazole treatment in CD. In 50% of the patients, GH values reach the range of controls. This could suggest that a relatively short-term decrease in circulating cortisol levels can partially restore glucocorticoid-induced GH suppression in CD. GH-releasing mechanisms stimulated by ghrelin/GHS could be more sensitive to the reduction in cortisol levels, as no changes in GHRH-induced GH release were observed. Further studies are necessary to clarify these hypotheses.

Declaration of interest

Funding

Acknowledgements

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