Association between tumoral GH-releasing peptide receptor type 1a mRNA expression and in vivo response to GH-releasing peptide-6 in ACTH-dependent Cushing’s syndrome patients

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Abstract

Objective: GH secretagogues (GHS) produce exaggerated ACTH and cortisol responses in Cushing’s disease (CD) patients, attributable to their direct action on GH-releasing peptide receptor type 1a (GHSR-1a). However, there are no studies correlating the in vivo response to GHS and GHSR-1a mRNA expression in ACTH-dependent Cushing’s syndrome (CS) patients. The aim of this study is to correlate the patterns of ACTH and cortisol response to GH-releasing peptide-6 (GHRP-6) to GHSR-1a expression in ACTH-dependent CS patients.

Design: Prospective study in a tertiary referral hospital center. Fifteen CD patients and two ectopic ACTH syndrome (EAS) patients were studied.

Methods: Tumor fragments were submitted to RNA extraction, and GHSR-1a expression was studied through real-time qPCR and compared with normal tissue samples. The patients were also submitted to desmopressin test and vasopressin receptor type 1B (AVPR1B) mRNA analysis by qPCR.

Results: GHSR-1a expression was similar in normal pituitary samples and in corticotrophic tumor samples. GHSR-1a expression was higher in patients (CD and EAS) presenting in vivo response to GHRP-6. Higher expression of AVPR1B was observed in the EAS patients responsive to desmopressin, as well as in corticotrophic tumors, as compared with normal pituitary samples, but no correlation between AVPR1B expression and response to desmopressin was observed in the CD patients.

Conclusions: Our results revealed a higher expression of GHSR-1a in the ACTH-dependent CS patients responsive to GHRP-6, suggesting an association between receptor gene expression and in vivo response to the secretagogue in both the CD and the EAS patients.
receptor expression might explain the dissimilar responses among patients. However, only few reports in the literature have attempted to correlate the desmopressin response pattern to the expression of vasopressin receptors. On the other hand, EAS patients have been reported to exhibit an in vivo response to desmopressin in up to 27% of cases (16, 17); also, association between the in vivo response and the expression of vasopressin receptors was demonstrated in some patients (13, 18, 19).

The aim of the present study was to correlate the in vivo ACTH and cortisol responses to GH-releasing peptide-6 (GHRP-6) to the quantitative expression of GHSR-1a mRNA in tumor samples from the ACTH-dependent CS patients. Patients were also submitted to the desmopressin test and AVPR1B mRNA analysis by quantitative PCR.

Subjects and methods

Patients and samples

Seventeen ACTH-dependent CS patients – 15 CD and 2 EAS (thymic carcinoid tumor and lung carcinoid tumor), median age 31 years (15–48), 13 females – were studied between April 2002 and August 2004 (Table 1). An informed consent form was signed, and the study was approved by the institutions’ Ethical and Research Committees.

The laboratory diagnosis of ACTH-dependent CS was confirmed by measurement of 24-h urinary cortisol (mean of three samples), loss of the circadian rhythm of cortisol secretion (midnight serum and salivary cortisol), lack of cortisol suppression with dexamethasone 1 mg, and ACTH plasma determination.

Differential diagnosis of ACTH-dependent CS work-up included the desmopressin and the GHRP-6 tests, pituitary magnetic resonance imaging (MRI), and computed tomography (CT) of abdomen, thorax, and cervical region. In the cases showing uncertain or negative MRI for pituitary tumor, bilateral, and simultaneous inferior petrosal sinus sampling was indicated. Central to peripheral ACTH gradient was defined as the ratio of plasma concentrations of ACTH O2 at basal conditions, and O3 after the desmopressin stimulation (20, 21).

Diagnosis was confirmed by histopathology of tumor (pituitary adenoma or carcinoid tumor) with positive immunostaining for ACTH. All pituitary tumors were evaluated by the same pathologist.

During the surgical procedure, tumor fragments were collected under sterile conditions and placed in 2.0 ml polypropylene vials containing 1.0 ml TRIzol (Invitrogen, Life Technologies), hermetically sealed and stored in liquid nitrogen for posterior study. Additional tumor fragments were sent for anatomicopathological and immunohistochemical analyses. All patients were
submitted to transsphenoidal pituitary surgery by the same neurosurgeon.

Autopsy tissue fragments (pituitary, thymus, and lung) with no evidence of pathologies were collected 8–12 h postmortem and used as controls. Tissues were taken from areas without hemorrhage or necrosis. The autopsy material was approved by the institutions’ Ethical and Research Committees and by the Death Verification Service of Sao Paulo.

**Desmopressin test protocol**

The desmopressin test was carried out in the morning, with patients in a resting supine position and under fasting conditions. Blood samples were collected before (−30, 0 min) and after (15, 30, 45, 60, and 90 min) an i.v. infusion of desmopressin 10 μg (DDAVP, Ferring Pharmaceuticals, Limhamn, Sweden) for ACTH and cortisol measurements. The response criteria were defined as an increment of >20% for cortisol and >50% for ACTH as compared with baseline values (22–24).

**GHRP-6 test protocol**

The GHRP-6 test was carried out in the morning, with patients in a resting supine position and under fasting conditions. Blood samples were collected before (−30, 0 min) and after (15, 30, 45, 60, and 90 min) an i.v. infusion of GHRP-6 1 μg/kg (Bachem California Inc., Torrance, California, USA) for ACTH and cortisol measurements. Criteria used for the GHRP-6 response were the same as for the desmopressin response, defined as an increment of >20% for cortisol and >50% for ACTH as compared with baseline values (22–24). Each patient was submitted to two tests, with a minimum interval of 48 h.

**Hormonal assays**

Serum cortisol was measured by fluorimunoassay in an AutoDELFIA System (Wallac Oy, Turku, Finland), with intra- and inter-coefficients of variation <10% and <12% (SI: 27.59 nmol/l; conversion factor: 27.59). ACTH was measured by an immunoradiometric method (CIS biointernational, Gié/Yvette, France), with intra- and inter-coefficients of variation <14% and <20% (SI: 2.2 pmol/l; conversion factor: 0.2202).

**RNA Isolation and cDNA Synthesis for PIT-1 expression evaluation**

Total RNA was isolated from fresh-frozen tissue samples of ACTH-secreting tumor and from postmortem normal pituitary (n = 7), thymus (n = 2), and lung (n = 1) samples using TRIzol reagent (Invitrogen, Life Technologies) according to the manufacturer’s instructions. The RNA quality was assessed by the integrity of rRNA bands (18S and 28S) following 1% agarose gel electrophoresis. Concentrations were quantified by spectrophotometry (GeneQuant Pro DNA/RNA Calculator, GE Healthcare Biosciences, Chalfont St Giles, Buckinghamshire, UK). Samples were kept at −80 °C until processing by quantitative PCR (qPCR). The cDNA was synthesized from 1 μg total RNA by SuperScript II (Invitrogen, Life Technologies) using random hexamers as primer. Reverse transcribed (RT)-PCR analysis for pituitary-specific transcription factor 1 (PIT1) was performed to indicate possible corticotrophic tumor contamination by normal pituitary tissue, as previously described (10). The breakpoint cluster region (BCR) (25) gene was co-amplified with PIT1 as internal control to assess the quality of cDNA preparations. Primer pairs were designed to have similar GC content and melting temperatures using Primer3 Program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3 www.cgi). Gene-specific primer sets were designed to amplify two separate intron-spanning regions that gave rise to products of 194 bp for PIT1 (sense 5'-GTG GGA GCA AAT GAA AGG AA-3' and antisense 5'-ACC CGT TTT TCT CTC TGC CTG CT-3' – GenBank accession no. NM_000306) and 377 bp for BCR (sense 5'-GAG AGG AGG GCG AAC AAG-3' and antisense 5'-CTC TGC TTA AAT CCA GTG GC-3' – GenBank accession no. BC066122). Reactions were made as standard for PCR amplification, using 0.4 μM of each BCR primer, 1.2 μM of each PIT1 primer, and Taq Paltinum DNA polymerase (Invitrogen, Life Technologies). The protocol for amplification was: denaturation for 30 s at 95 °C, annealing for 1 min at 57 °C, and extension for 1 min and 30 s at 72 °C, during 35 amplification cycles. In each PCR, a negative control in which the template was omitted was run. The PCR products were analyzed on agarose gels stained with ethidium bromide, for visualization of the appropriate bands (377 and 194 bp for BCR and PIT1 respectively).

**GHSR-1α and AVPR1B quantitative PCR analysis**

Transcript levels of GHSR-1α and AVPR1B were determined as the number of transcripts relative to those of GAPDH and additionally normalized to the mean value of control pituitary. Quantitative PCR (qPCR) analyses of all tumor samples and normal tissue samples were performed in the Rotor-Gene RG-3000 (Corbett Research, Sidney, Australia) using Quantitect SYBR Green RT-PCR for quantitative, real-time, one-step RT-PCR (Qiagen GmbH), according to the manufacturer’s instructions. Reactions were made by combining 12.5 μl SYBR RT-PCR Master Mix, 0.25 μl QuantiTect RT Mix, 0.2 μM sense/antisense primers, and 5 μl (20 ng/μl) total RNA template. The RNA template concentrations (100, 50, 25, 12.5, 6.5, and 3.2 ng/μl) were used to generate a standard curve, in order to evaluate the amplification efficiency of each target gene as compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The reaction was carried out under the following cycling conditions: 50 °C for 30 min for RT, heated to 95 °C for
15 min, and then cycled 35 times at 94 °C for 20 s, 60 °C for 30 s (GAPDH), 53 °C (AVPR1B) or 58 °C (GHSR-1a) for 30 s, and 72 °C for 30 s. To verify the amplification reaction accuracy, melting curve analysis was performed using the following parameters: 72 °C ramping to 99 °C at 0.2 °C/s. The threshold cycle (Cₜ) value is taken as the fractional cycle number at which the emitted fluorescence of the sample passes a fixed threshold above the baseline. All primer pairs were designed as previously described, on two adjacent exons to achieve a high level of specificity and also to avoid detection of genomic DNA: AVPR1B (sense 5'-CAG CAG CAT CAA CAC CAT CT-3' and antisense 5'-CCA TGT AGA TCC AGG GGT TG-3' – GenBank accession no. NM_000707); GHSR-1a (sense 5'-ACC AGA ACC ACA AGC AAA CC-3' and antisense 5'-TGA TGG CAG CAC TGA GGT AG-3' – GenBank accession no. NM_198407); GAPDH (sense 5'-GAA GAT GGT GAT GGG ATT TC-3' and antisense 5'-GAA GGT GAA GGT CGG AGT-3'). Specificity of amplicons was also ensured by agarose gel electrophoresis to visualize a unique product fragment for AVPR1B, GHSR-1a, and GAPDH with appropriate size (221, 192, and 226 bp respectively). The RNA content of samples was normalized using the expression level of GAPDH and compared with control pituitary. All samples were analyzed in duplicate. Relative quantification was calculated using the 2^ΔΔCt (26) method for the AVPR1B gene, based on their equivalent amplification efficiency for GAPDH. For GHSR-1a, whose amplification efficiency for the control gene (GAPDH) was different, the relative quantification was calculated using the mathematical model described by Pfaffl (27).

**Statistical analysis**

The statistical analysis was carried out using the software JMP Starter 5.1.1. (SAS Institute Inc., Heidelberg, Germany). The Mann–Whitney test was used for the analysis of ACTH and cortisol responses in dynamic tests. The median test was used to compare results on GHSR-1a and AVPR1B mRNA expression in tumor and normal tissue samples. The Kruskal–Wallis test was used to analyze gene expression data when more than two groups were compared. Statistical significance was set at P value < 0.05. Values are provided as means ± s.d.

**Results**

**GHSR-1a mRNA expression and association with in vivo response to GHRP-6**

Samples from the CD patients, not contaminated by normal pituitary tissue (PIT1 negative), were used for the study of GHSR-1a and AVPR1B mRNA expression, with a total of 11 corticotrophic tumor and 2 ectopic ACTH-producing tumor samples. Four PIT1-positive corticotrophic tumor samples were excluded from the molecular studies (Table 1).

The CD patients (n = 15) presented a significant increase in cortisol and ACTH levels after the GHRP-6 infusion (basal versus peak: 21 ± 6.4 to 31 ± 10.6 µg/dl, SI: 579.4 ± 176.6 to 855.3 ± 292.4 nmol/l, P < 0.001; 104.7 ± 106.5 to 317.8 ± 252.2 mg/ml, SI: 23 ± 23.4 to 70 ± 55.5 pmol/l, P < 0.001; respectively). When patients were individually analyzed, 66.7% (10 out of 15) exhibited positive responses to the test (Table 1). Concerning EAS patients, no significant cortisol response to GHRP-6 was observed (basal versus peak: 36.3–38.5 µg/dl, SI: 1001.5–1062.2 nmol/l, 6% (thymic carcinoid tumor); and 61.4–68.3 µg/dl, SI: 1694–1884.4 nmol/l, 11.2% (lung carcinoid tumor)). However, one patient (lung carcinoid tumor) presented a high and significant plasma ACTH response (883–1545 pg/ml, SI: 194.4–340.2 pmol/l, 75%), whereas the other patient was non-responsive (433–371 pg/ml, SI: 95.3–81.7 pmol/l; Fig. 1). A possible explanation for the discordant results of ACTH and cortisol response to GHRP-6 in the lung carcinoid tumor patient would be the incapacity of the adrenal glands to additionally secrete cortisol under overstimulation conditions.

Although not significant, a tendency toward higher expression of GHSR-1a was observed in corticotrophic tumor samples (n = 11) as compared with normal pituitary samples (Fig. 2A). Nevertheless, considering the outcome of the GHRP-6 test in the CD patients, a higher mRNA expression of GHSR-1a was observed among responsive subjects (P = 0.023), thus establishing an association between the in vivo response and the magnitude of GHSR-1a expression (Fig. 2B).

Similarly, an association between the in vivo response to GHRP-6 and the intensity of GHSR-1a mRNA expression was demonstrated in our two EAS patients. The sample from the lung carcinoid tumor patient presenting significant ACTH response to GHRP-6 showed intense GHSR-1a mRNA expression as compared with both the normal lung sample and the thymus sample from the EAS patient who was not responsive to GHRP-6. The magnitude of GHSR-1a mRNA expression in the former patient was comparable with corticotrophic tumors and was higher than in normal pituitary samples (Fig. 3A).

**AVPR1B mRNA expression and association with in vivo response to desmopressin**

The CD patients (n = 15) presented a significant increase in cortisol and ACTH levels after the desmopressin infusion (basal versus peak: 22.7 ± 7.9 to 34 ± 10.8 µg/dl, SI: 626.3 ± 218 to 938 ± 298 nmol/l, P < 0.001; 94.2 ± 76.7 to 303.8 ± 327 pg/ml, SI: 20.7 ± 16.9 to 66.9 ± 72 pmol/l, P < 0.001; respectively). When patients were individually analyzed,
73.3% (11 out of 15) exhibited responses to the test (Table 1). On the contrary, EAS presented no significant cortisol response to desmopressin (basal versus peak: 40.2–47.8 μg/dl, SI: 1109.1–1318.8 nmol/l, 18.9% (thymic carcinoid tumor); and 68.6–70.3 μg/dl, SI: 1892.6–1939.6 nmol/l, 2.4% (lung carcinoid tumor)). Nevertheless, a significant plasma ACTH response was observed in the patient harboring a thymic carcinoid tumor (360–1326 pg/ml, SI: 79.3–292 pmol/l, 268%), but no response was observed in the patient harboring a lung carcinoid tumor (510–611 pg/ml, SI: 112.3–134.5 pmol/l, 19.8%) (Fig. 1).

A higher mRNA expression of AVPR1B was observed in corticotrophic tumors (n=11) as compared with normal pituitary samples (P=0.018) (Fig. 4A). However, when CD was analyzed according to responsiveness versus non-responsiveness to desmopressin, we did not observe an increased expression of AVPR1B mRNA among the responsive ones (Fig. 4B).

Also the association between in vivo response to desmopressin and the intensity of AVPR1B mRNA expression in EAS was interesting. Molecular analysis from the thymic carcinoid tumor in the patient who presented a significant response to desmopressin showed intense AVPR1B mRNA expression as compared with both normal thymus samples and the sample from the lung carcinoid tumor patient who did not show response (Fig. 3B).

**Discussion**

The mechanism of the exaggerated responses of ACTH and cortisol to GHS in the CD patients is not fully understood. There is evidence of direct action of GHS on corticotrophic tumors, probably mediated by active GHS receptor – GHSR-1a. However, no direct effect was observed in a study with cultures of corticotrophic adenomas evaluated by intracellular calcium flow (28), while a direct action of ghrelin on two corticotrophinoma culture cells was recently demonstrated (29).
In normal tissues, GHSR-1a is expressed mainly in the hypothalamus and pituitary (30). In pituitary tumors, GHSR-1a is expressed mostly in somatotrophinomas, although all histological subtypes of pituitary adenomas can express this receptor (10). Moreover, other neuroendocrine tumors such as carcinoid tumors commonly show GHSR-1a expression, even in the absence of CS (9, 10, 31).

Previous investigations on GHSR-1a mRNA expression in corticotrophic tumors showed discordant results when compared with normal pituitary tissues. These results might be due to some limitations of those studies, such as the use of primers unable to identify exactly which GHS receptor (‘GHSR’) was being studied, the small number of samples, and the use of less accurate methodologies (semi-quantitative RT-PCR) (9, 10). Employing semi-quantitative RT-PCR, De Keyzer et al. reported an increased expression of GHSR-1a in the CD patients (9). In a more recent report – the only one using quantitative PCR – Korbonits et al. showed a similar expression of this receptor in corticotrophic tumors and normal pituitary tissue, a result that is comparable with those observed in our series (10).

Nonetheless, our study demonstrated a higher expression of GHSR-1a mRNA in samples from the GHRP-6-responsive CD patients as compared with non-responders. Although the expression of GHSR-1a was similar in normal pituitary tissue and in corticotrophic tumor samples as a whole, a higher expression as compared with normal pituitary tissue could be observed when only responsive cases were analyzed. In addition, the results from our EAS patients suggest an association between the in vivo response and GHSR-1a mRNA expression. Further studies of EAS patients are required in order to confirm this association. Therefore, the pattern of ACTH in vivo response to GHS in the ACTH-dependent
CS patients can be explained by tumor GHSR-1a expression. It must be emphasized that our study has the limitation of not having used techniques that might confirm protein expression (immunohistochemical test or Western blot). Further studies are necessary to confirm such expression. However, other mechanisms can be involved in these responses. Recently, a study demonstrated co-localization of ACTH and ghrelin in the same granules of two densely granulated corticotrophic tumors. The authors suggested that autocrine or paracrine action of ghrelin could be involved in the modulation of ACTH secretion by tumors (29).

Regarding the physiopathology of GHSR-1a expression in the ACTH-dependent CS patients, it should be mentioned that increased expression of this receptor was hypothesized to be either primary or induced by hypercortisolism. An animal model has shown that adrenalectomy significantly reduced the expression of GHSR in the pituitary, and that replacement therapy with dexamethasone re-established this expression (32). Also, a recent report evaluating the promoter region of the human GHSR-1a gene, which includes a region responsive to glucocorticoids, showed a negative gene transcription regulation to glucocorticoids (33).

The pattern of exaggerated ACTH and cortisol response to desmopressin in the ACTH-dependent CS patients has been attributed to a corticotrophic ‘overexpression’ of AVPR1B and AVPR2 (13–15, 18, 19, 34). On the other hand, no mutations in the AVPR1B receptor have been detected in the CD patients (14).

The present study shows a probable association between AVPR1B mRNA expression and in vivo response to desmopressin in EAS patients. In the thymic carcinoid tumor patient who responded to desmopressin, AVPR1B mRNA expression was increased as compared with normal thymus sample and to the sample from the non-responsive lung carcinoid tumor patient. Other EAS patients must be studied to confirm the association between AVPR1B expression and in vivo response to desmopressin. Studies analyzing this clinical-molecular correlation in CD are scarce. In a paper by Dahia et al., among 11 cases presenting significant expression of AVPR1B, only 1 was submitted to the desmopressin test, and it was responsive (14). In recently published data, no correlation was established between the in vivo response to desmopressin in the CD patients and the in vitro responses of primary cultures of corticotrophic tumors from the same patients suggesting that ACTH secretion may be mediated by extra-pituitary factors (35). In our series, an increased expression of AVPR1B mRNA was observed in tumor samples from the CD patients as compared with normal samples, as had been shown in previous reports (13–15). Nonetheless, we found no differences in AVPR1B expression when the CD patients were divided into responders and non-responders to the desmopressin test, possibly due to the small number of non-responders. It should be stressed that the majority of these patients responded to the test and presented overexpression of this receptor.

In conclusion, higher GHSR-1a mRNA expression was observed in responsive CD and EAS patients for the GHRP-6 test, suggesting an association between receptor expression and in vivo response to the secretagogue. An association between the desmopressin response and the AVPR1B mRNA expression is suggested in EAS patients but not in the CD patients. In spite of the small number of EAS patients, the aforementioned positive responses in EAS patients limit the use of these secretagogues for the differential diagnosis of ACTH-dependent CS.

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Figure 4 AVPR1B mRNA expression. (A) Corticotrophic tumor (n=11) and normal pituitary samples and (B) desmopressin respondent and non-respondent CD patients. CD, Cushing’s disease; NP, normal pituitary; *mathematical model of 2^−ΔΔC_T (26).

The horizontal line within the box plot diagram represents the median value, the box encompasses the 25th–75th percentiles, and the error bars include the 10th–90th percentiles.
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