Analysis of glucose-dependent insulinotropic peptide receptor (*GIPR*) and luteinizing hormone receptor (*LHCGR*) expression in human adrenocortical hyperplasia

Análise da expressão dos receptores do peptídeo insulinotrópico dependente de glicose (*GIPR*) e do hormônio luteinizante (*LHCGR*) nas hiperplasias adrenocorticais humanas

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ABSTRACT

Objective: To analyze the aberrant expression of the *GIPR* and *LHCGR* in different forms of adrenocortical hyperplasia: ACTH-independent macronodular adrenal hyperplasia (AIMAH), primary pigmented nodular adrenocortical disease (PPNAD) and diffuse adrenal hyperplasia secondary to Cushing's disease (DAHCD). **Methods:** We quantified *GIPR* and *LHCGR* expressions using real time PCR in 20 patients with adrenocortical hyperplasia (seven with AIMAH, five with PPNAD, and eight with DAHCD). Normal adrenals tissues were used as control and the relative expression was compared with β-actin. **Results:** *GIPR* and *LHCGR* expressions were demonstrated in all tissues studied. Median *GIPR* and *LHCGR* mRNA levels were 1.6; 0.4; 0.5 and 1.3; 0.9; 1.0 in adrenocortical tissues from AIMAH, PPNAD and DAHCD respectively. There were no differences between *GIPR* and *LHCGR* expressions in all tissues studied. **Conclusions:** *GIPR* and *LHCGR* overexpression were not identified in the studied cases, thus suggesting that this molecular mechanism is not involved in adrenocortical hyperplasia in our patients. Arq Bras Endocrinol Metab. 2009;53(3):326-31.

Keywords
Adrenal hyperplasia; gene expression; G-protein coupled receptors (GPCRs)

RESUMO

Objetivo: Analisar a expressão aberrante do *GIPR* e do *LHCGR* em diferentes formas de hiperplasias adrenocorticais: hiperplasia adrenal macronodular independente de ACTH (AIMAH), doença adrenocortical nodular pigmentada primária (PPNAD) e hiperplasia adrenal difusa secundária à doença de Cushing (DAHCD). **Métodos:** Quantificou-se por PCR em tempo real a expressão desses receptores em 20 pacientes: sete com AIMAH, cinco com PPNAD e oito com DAHCD. Adrenais normais foram utilizadas como controle e a expressão relativa desses receptores foi comparada à expressão da β-actina. **Resultados:** A expressão desses receptores foi demonstrada em todos os tecidos estudados. A mediana da expressão do *GIPR* e do *LHCGR* foi de 1,6; 0,4; 0,5 e de 1,3; 0,9; 1,0 nos tecidos dos pacientes com AIMAH, PPNAD e DAHCD, respectivamente. Não houve diferença significativa na expressão desses receptores nos tecidos estudados. **Conclusões:** Hiperexpressão do *GIPR* e do *LHCGR* não foi observada, sugerindo que esse mecanismo não está envolvido na patogênese molecular da hiperplasia adrenal nesses pacientes. Arq Bras Endocrinol Metab. 2009;53(3):326-31.

Descritores
Hiperplasia adrenal; expressão gênica; receptores de membrana

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INTRODUCTION

ACTH-independent Cushing's syndrome may occur due to adrenocortical tumors and different kinds of hyperplasia: ACTH-independent macronodular adrenal hyperplasia (AIMAH), primary pigmented nodular adrenocortical disease (PPNAD) and its variant subtype, non-pigmented micronodular hyperplasia (1-3).

The pathways involved in ACTH-independent hormone secretion and cell proliferation in these disorders have not been completely elucidated (4-6). The cortisol production in AIMAH has been shown to be regulated by eutopic and ectopic aberrant expression of G-protein coupled receptors (1,2,7-9).

In PPNAD, presented frequently as part of Carney complex syndrome (10), germline mutations of *PRKAR1A* and, recently, *PDE11A* mutations were related to the etiology of the disease (11,12).

The aberrant expression of G-protein coupled receptor has been related to some cases of adrenal hyperplasia. The glucose-dependent insulinotropic peptide receptor (GIPR), a member of the secretin-vasoactive intestinal peptide receptor family, is widely distributed in peripheral organs as well as in the brain (13). During the last decade, GIPR overexpression has been identified in the adrenals of patients with Cushing's syndrome due to AIMAH and adrenal adenoma (2,14-18). Its expression has also been described in adrenal hyperplasia secondary to Cushing's disease, however, the form how GIPR overexpression stimulates the steroidogenesis pathway remains uncertain (19,20).

The luteinizing hormone receptor (LHCGR), a G protein-coupled receptor mainly involved in the regulation of gonadal functions (21), is normally expressed in the human zona reticularis of the adrenal gland (22). The aberrant *LHCGR* adrenal expression was first identified in a French-Canadian woman with transient Cushing's syndrome during pregnancies that reappeared after post-menopausal LH increase (23). Overexpression of this receptor was then identified in several *in vitro* studies of steroid-secreting AIMAH and adrenocortical tumors (24-26).

The aim of this study is to investigate, by using real time PCR, if the aberrant expression of these receptors, *GIPR* and *LHCGR*, would be involved in adrenal enlargement in PPNAD and Cushing's disease, as well as in our cases of AIMAH.

METHODS

The study was approved by the Ethical Committee of Hospital das Clínicas, São Paulo, Brazil, and written informed consent was obtained from all patients. We studied 20 Brazilian patients with adrenocortical disorders (18 females and 2 males; age ranged from 18 to 69 years old). Seven patients had AIMAH, five had PPNAD and eight patients had Cushing's disease. Complete clinical and molecular features of these patients are shown in table 1. The pre-surgical hormonal evaluation of the patients included peripheral blood determination of electrolytes, LH, FSH, testosterone, estradiol, ACTH, dehydroepiandrosterone sulfate (DHEAS), dehydroepiandrosterone (DHEA), androstenedione, 11-deoxycortisol, aldosterone, plasmatic renin activity, cortisol levels in basal condition and after overnight administration of 1 and 8 mg of dexamethasone. Urinary cortisol of 24 hours was also determined.

Six of seven cases of AIMAH (patients 2-7, Table 1) were previously submitted to an *in vivo* screening protocol for the aberrant receptor presence (27) and two siblings (cases 5 and 7) have presented a cortisol increment > 50% after cisapride test, suggesting an abnormal response due to 5-HT4 receptor.

Quantitative expression of *GIPR* and *LHCGR*

All patients underwent bilateral or unilateral adrenalectomy, except for two patients (cases 5 and 6 of Table 1), in whom adrenal biopsies were performed. Adrenal tissue was obtained after surgical proceedings. Tumor samples were obtained from the core of the excised tumors to minimize possible contamination by the surrounding normal tissue. Necrotic and hemorrhagic areas were also avoided. Nodule tissue fragments were immediately stored in liquid nitrogen until RNA extraction.

RNA extraction, DNA synthesis and RT-PCR

Total RNA was isolated from frozen tissue using Trizol Reagent (Invitrogen, Grand Island, NY, USA). Reverse transcription (RT) was performed in 5 µg of total RNA of each sample using Multiscribe from a High-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) in a 50 µL total reaction.

Quantitative real time PCR was carried out in the Applied Biosystems 7000 real-time PCR System. TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) were especially designed to amplify

Table 1. Clinical and molecular data of 20 patients with adrenocortical hyperplasia

Patients	Age (years)	Sex	Phenotype	Genetic alterations	Diagnosis	GIPR *Expression	LHCGR *Expression
1	34	F	Cushing's syndrome	-	AIMAH	138.5	0.9
2	53	F	Cushing's syndrome	-	AIMAH	13	1.3
3	26	F	Cushing's syndrome	-	AIMAH	1.6	3.0
4	69	F	Cushing's syndrome	-	AIMAH	0.8	1.3
5	51	F	Cushing's syndrome	-	AIMAH	0.6	0.8
6	45	F	Cushing's syndrome	-	AIMAH	3.0	2.1
7	44	M	Cushing's syndrome	-	AIMAH	0.2	0.05
8	18	M	Cushing's syndrome	Y21X(*PRKAR1A*)	PPNAD	0.2	0.9
9	55	F	Cushing's syndrome	-	PPNAD	0.05	0.4
10	29	F	Cushing's syndrome	-	PPNAD	0.4	0.9
11	35	F	Cushing's syndrome	-	PPNAD	0.5	1.8
12	23	F	Cushing's syndrome	-	PPNAD	0.4	2.1
13	31	F	Cushing's syndrome		DAHCD	0.3	0.5
14	34	F	Cushing's syndrome		DAHCD	0.9	0.7
15	35	F	Cushing's syndrome		DAHCD	0.7	0.7
16	32	F	Cushing's syndrome		DAHCD	0.5	0.8
17	31	F	Cushing's syndrome		DAHCD	0.2	1.1
18	27	F	Cushing's syndrome		DAHCD	1.5	1.3
19	33	F	Cushing's syndrome		DAHCD	0.3	1.4
20	32	F	Cushing's syndrome		DAHCD	0.4	1.7
			Normal adrenal			0.9	1.3
			Normal adrenal			0.9	1.5
			Normal adrenal			0.9	1.8
			Normal adrenal			0.5	1.9
			Normal adrenal			1.4	3.0
			Normal adrenal			0.4	3.4
			Normal adrenal			0.1	3.7
			Normal adrenal			0.5	9.6
			Control tissue (pancreas)			74.4	
			Control tissue (testes)				45.3

F: female; M: male; DAHCD: diffuse adrenal hyperplasia secondary to Cushing disease.
* Relative expression levels compared to β-actin.

the *LHCGR*. The *GIPR* amplification was performed using available commercial primers and a probe (Assay ID Hs006092_m1, Applied Biosystems Foster City, CA, USA). The *LHCGR* amplification was performed with the following pair of primers, 5' GCACAATGGAGCCTTCCGT 3'; 5' GGCCTGCAATTTGGTGGAA 3' and the probe 5' CCGAAAACCTTGGATATTT 3'. β-actin (assay ID-4326315E, Applied Biosystems Foster City, CA, USA) was chosen as the internal control. Multiplex reactions consisted of 12.5 µL 2x TaqMan Universal PCR master mix, 1.25 µL of each 20x assay on demand, 1.5 µL of cDNA and water to complete 25 µL final volume. PCR parameters were 50°C for two minutes, 95°C for ten minutes followed by 50 cycles at 95°C for 15 seconds and 60°C for 1 minute.

Validation experiments were performed to verify that the amplification efficiency of the controls was similar to that of the target genes.

A cycle threshold (C_T) value in the linear range of amplification was selected for each sample in triplicate and normalized to β-actin expression levels. The relative expression levels were analyzed using the $2^{-\Delta\Delta CT}$

where the $\Delta\Delta C_T$ is the difference between the selected ΔC_T value of a particular sample and the ΔC_T of a pool using 61 normal adrenals from autopsies (Clontech, Palo Alto, CA, USA) (28). The mean expression of the target genes in the normal adrenals pool was assigned an expression value of 1.0 and the fold increase or decrease in the expression levels in each hyperplasia sample was determined by comparison.Pancreas and testis were obtained during the surgical resections of kidney tumors, pancreatic cysts and gonads, being used as positive expression controls for *GIPR* and *LHCGR*, respectively.

Statistical analysis

GIPR and *LHCGR* expressions from all tissues analyzed were compared by Kruskal Wallis test. The value of $p < 0.05$ was considered statistically significant. In each group of patients, data are presented as median and range. The Spearman test was used to establish correlation between the receptor expression, clinical aspects and hormonal levels of patients.

RESULTS

The expression of *GIPR* and *LHCGR* was demonstrated in all tissues studied. *GIPR* expression was very low in all normal adrenal tissues studied (median level: 0.7, ranging from 0.1 to 1.4) while *LHCGR* expression levels in normal adrenal tissues were more variable (median level: 2.5, ranging from 1.3 to 9.6). Median *GIPR* mRNA levels were 1.6, 0.4 and 0.5 in adrenocortical tissues from patients with AIMAH, PPNAD and Cushing's disease respectively (Figure 1). Median *LHCGR* mRNA levels were 1.3, 0.9 and 1.0 in adrenocortical tissue from patients with AIMAH, PPNAD and Cushing's disease respectively (Figure 2). The median and ranges of both receptor expressions are shown in table 2.

No significant difference in *GIPR* expression was observed among these forms of adrenocortical hyperplasia and normal adrenals, while it was observed that the *LHCGR* expression was lower in AIMAH ($p = 0.02$) and Cushing's disease ($p = 0.02$), but not statistically significant in PPNAD when compared to normal adrenals ($p = 0.06$). We did not observe any statistical difference between *GIPR* and *LHCGR* expressions in the different forms of hyperplasia studied (Table 2).

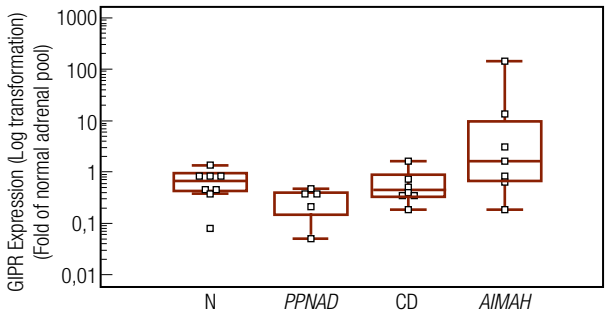

Figure 1. Expression levels of *GIPR* mRNA of 20 adrenal hyperplasia cases: five PPNAD, eight hyperplasia cases secondary to CD (Cushing's disease) and seven AIMAH compared to eight normal adrenals.

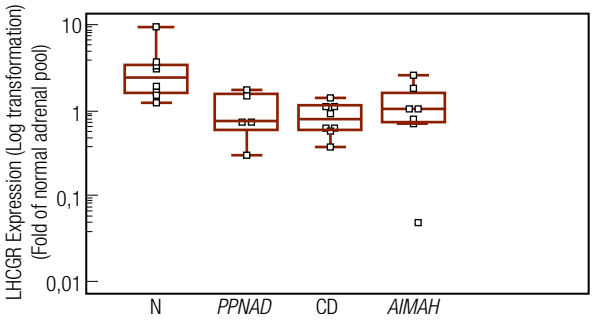

Figure 2. Expression levels of *LHCGR* mRNA of 20 adrenal hyperplasia cases: five PPNAD, eight hyperplasia cases secondary to CD (Cushing's disease) and seven AIMAH, compared to eight normal adrenals.

Table 2. *GIPR* and *LHCGR* relative expression levels (median and range) in adrenocortical hyperplasia

	GIPR Expression	LHCGR Expression
AIMAH	1.6 (0.2 - 138.5)	1.3 (0.05 - 3.0)
PPNAD	0.4 (0.05 - 0.5)	0.9 (0.4 - 2.1)
DAHCD	0.5 (0.2 - 1.5)	1.0 (0.5 - 1.7)
Normal adrenal	0.7 (0.1 - 1.4)	2.5 (1.3 - 9.6)

DAHCD: diffuse adrenal hyperplasia secondary to Cushing's disease.

We did not find a correlation between GIPR and LHCGR expression levels in adrenocortical tissues and pre-surgical hormonal levels ($p > 0.05$).

DISCUSSION

Cushing's syndrome secondary to aberrant hormone receptors has been described by several authors in the last decade (7,29). This condition has been largely identified in AIMAH and adenomas, and some sporadic cases of hyperplasia secondary to Cushing's disease and PPNAD were also related to this mechanism (2,8,19). Recently,

new patterns of LHCGR and GIPR expressions have been described, implicating both receptors in the pathophysiology of aldosterone-secreting tumors and androgen secretion resulting in hirsutism, suggesting a larger role of G-protein receptors in adrenocortical disease (26,30).

To investigate the potential role of GIPR and LHCGR expression in adrenocortical hyperplasia we studied a group of patients (20 cases) with adrenal enlargement due to several etiologies.

Despite the molecular mechanism responsible for the aberrant expression of these receptors, they still need to be clarified (2,31-34); experimental studies have demonstrated that abnormal expression of GIPR and LHCGR in adrenocortical cells provoke phenotypic changes in these cells, leading to the deregulation in their proliferation fate and eliciting adrenocortical tumorigenesis. This hyperproliferative adrenocortical tissue lead to GIP or LH-dependent secretion of cortisol and low ACTH levels (35,36).

The abnormal adrenal expression of the functional LHCGR has been identified in some cases of AIMAH and adrenocortical tumors (23-25,37); this receptor expression has been documented in normal adrenal gland (38), although it was not well investigated in other forms of adrenocortical hyperplasia; We therefore examine the LHCGR expression in eight cases of adrenocortical hyperplasia secondary to Cushing's disease and five cases of PPNAD that have presented lower LHCGR expression when compared to normal adrenal tissues. Our results do not support the role of this receptor in the adrenal enlargement due to such disorders.

The hypothesis that chronic stimulation or activation of the ACTH signaling pathway may be associated to GIPR expression suggested by the results of Swords and cols. (19) in all five patients with diffuse adrenocortical hyperplasia secondary to Cushing's disease and one case with PPNAD has not been confirmed (20,39). In our study, no difference in GIPR expression was observed in the different forms of adrenocortical hyperplasia studied: adrenal hyperplasia secondary to Cushing's disease; AIMAH and PPNAD, as well as between adrenocortical hyperplasia and normal adrenals, which did not confirm the previously described findings. However, the patient 1 showed a high expression level (138.5) bringing into evidence the great variability of GIPR expression in the adrenal hyperplasia (40,41).

In conclusion, we ruled out *GIPR* and *LHCGR* overexpression as being related to adrenocortical hyperplasia due to AIMAH and PPNAD in our cases. Our results suggest that GIPR is not involved in the molecular mechanisms implicated in the development of diffuse adrenocortical hyperplasia of Cushing's disease. These data support the idea that the role of ACTH stimulation in the regulation of GIPR ectopic expression might be really reduced.

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