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Cushing's Syndrome in a Patient with Bilateral Macronodular Adrenal Hyperplasia Responding to Cisapride: An *in Vivo* and *in Vitro* Study

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Cortisol secretion in adrenal Cushing's syndrome can be regulated by aberrant hormone receptors, such as gastric inhibitory polypeptide, V1 vasopressin, catecholamines, LH/human chorionic gonadotropin, and serotonin receptors. We report the case of a patient with Cushing's syndrome due to bilateral adrenal macronodular hyperplasia. Extensive *in vivo* testing for the presence of aberrant receptors revealed a 5-fold increase of plasma cortisol after the administration of cisapride, an agonist of the serotonin 4 (5-HT_4) receptor. Primary cell cultures were established from adrenocortical specimens obtained at surgery, and *in vitro* studies also showed that cisapride determined an increase [133.7 ± 5.5% (mean ± SE) of baseline, considered 100%) of cortisol secretion from cultured cells. The presence of 5-HT_4 receptor transcript, and in particular of isoforms c, g, and n, was confirmed by RT-PCR, and the determination of the mRNA levels by real-time RT-PCR revealed a higher expression than in normal adrenal glands. To our knowledge, this is one of the first reports of Cushing's syndrome in which cortisol secretion is regulated mainly by the 5-HT_4 receptor, among known aberrant receptors. In addition, it is noteworthy that hypocortisolism ensued after the removal of the most enlarged adrenal gland, but the *in vivo* response to cisapride persisted. (*J Clin Endocrinol Metab* 88: 4616–4622, 2003)

ENDOGENOUS CUSHING'S SYNDROME is attributable to a primary adrenal pathology in about 20% of cases (1, 2). Among these, bilateral adrenal hyperplasia is responsible for approximately 10% of the syndrome. Mutations of the gene encoding the protein kinase A type I-α regulatory subunit have been demonstrated recently in patients with bilateral pigmented micronodular adrenal hyperplasia (3). Although the pathophysiology of endogenous hypercortisolism, especially in cases of ACTH-independent unilateral adrenal adenoma or bilateral macronodular adrenal hyperplasia (AIMAH), has been only partially unraveled until recently (1, 2), it is undoubtedly becoming better understood. AIMAH is very often characterized by the presence of illicit hormone receptors on the adrenocortical cells, as suggested by the abnormal *in vivo* cortisol secretion evoked by substances that do not cause any plasma cortisol increase in normal subjects (4). Several types of adrenal illicit receptors have been described in primary adrenal Cushing's syndrome; among these are receptors for gastric inhibitory polypeptide, V1-vasopressin receptors, β-adrenergic receptors, LH/human chorionic gonadotropin (hCG) receptors, and serotonin 4 (5-HT_4) receptors (4). Adrenal illicit 5-HT_4 receptors have been found, by means of cortisol response to cisapride or metoclopramide in patients with AIMAH and subclinical or overt Cushing's syndrome, in association with other aberrant receptors (*i.e.* LH/hCG, V1-vasopressin, β-adrenergic receptors) (5–8). In only one patient has the exclusive presence of the 5-HT_4 receptor, among known illicit adrenal receptors, been described so far (7). The demonstration that the presence and activation of adrenal illicit receptors may be responsible for chronic hypercortisolism has been provided in some cases in which remission of the disease was observed under specific receptor blockade (5, 9). With regard to the 5-HT_4 receptor, it is worth mentioning that nine different isoforms have been described so far (10–13), and in a recent study isoform characterization and quantitative determination of 5-HT_4 receptor mRNA in AIMAH have been performed (7). Interestingly, 5-HT_4 receptor overexpression was found in four of six cases. On the other hand, in the two remaining cases, the expression level was similar to that observed in the normal adrenal cortex, and isoforms present in normal glands were also detected. Furthermore, no gain-of-function mutations of the 5-HT_4 receptors were found in these two cases in which the enhanced sensitivity to 5-HT_4 receptor agonists remains unclear (7). In this paper, we report the case of a patient affected by Cushing's syndrome due to AIMAH who showed a clear increase in plasma cortisol levels only after cisapride administration during the screening for illicit receptors, suggesting at first sight the involvement of 5-HT_4 in the pathogenesis of her disease.

Patient and Methods

Patient

A 58-yr-old woman was referred to our unit for a clinical syndrome characterized by weight gain, fatigue, muscle weakness, and hypertension. An abdominal ultrasound scan, performed after the finding of an altered value of γ-glutamyl transferase, had revealed the presence of bilateral adrenal enlargement.

The clinical history of the patient was characterized by a hysterectomy performed after her third pregnancy, when she was 36 yr old, and by frequent episodes of bronchial asthma and an external quadrantec-

Abbreviations: AIMAH, ACTH-independent macronodular adrenal hyperplasia; AT-1, angiotensin II type 1; AVP arginin-vasopressin; CT, computed tomography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hCG, human chorionic gonadotropin; 5-HT_4, serotonin 4.

tomy of her right mammary gland for a differentiated ductal carcinoma (G1 Bloom-Richardson), performed 4 months before her admission. For this disease, the patient was receiving local radiotherapy and medical treatment with tamoxifen when evaluated.

Physical examination revealed central obesity with cervical buffalo hump, a rounded face, thin skin with bruises, a marked dorsal scoliosis, and marked muscle hypotrophy of the limbs. Body weight was 63 kg, height was 158 cm, blood pressure was 170/100 mm Hg, and heart rate was 100 beats per minute.

Initial investigation showed an increase in urinary free cortisol, with 444 and 373 nmol/24 h on 2 different days (normal values, <275 nmol/24 h). Circadian rhythm of cortisol was absent, as shown by the following values: 671 nmol/liter at 0800 h, 668 at 1200 h, 721 at 1600 h, 774 at 2000 h, and 656 at 2400 h. Basal plasma ACTH was 10.5 ng/liter (normal range, 9–52 ng/liter). Plasma concentration of dehydroepiandrosterone sulfate was below 0.4 μmol/liter (normal range, 0.4–5.4 μmol/liter); testosterone was 1.7 nmol/liter (normal range, 0.5–3.0 nmol/liter); Δ-4-androstenedione, 1.4 nmol/liter (normal range, 1.4–9.4 nmol/liter); and 17-hydroxy-progesterone, 0.52 nmol/liter (normal range, 1.2–8.0 nmol/liter). Blood electrolytes and fasting and postprandial glucose were normal, triglycerides were elevated (350 mg/dl; normal range, 50–170 mg/dl). Plasma ACTH and cortisol were not suppressed after an overnight administration of either 2 mg (10.6 ng/liter and 501 nmol/liter, respectively) or 8 mg (10.4 ng/liter and 875 nmol/liter, respectively) dexamethasone.

An abdominal computed tomography (CT) scan confirmed the bilateral adrenal macronodular hyperplasia with a predominant 7.4 × 3.9-cm solid, hypodense nodule in the left gland and a similar 4.0 × 2.1-cm nodule in the right gland. Hounsfield units were 18 on unenhanced CT scan and 32 on enhanced CT scan.

An ^{131}I-cholesterol scan showed an intense uptake of the tracer after 24 h in the left adrenal and a less intense uptake after 48 h in the contralateral gland.

After 1 month of therapy with ketoconazole (200 mg twice a day), the patient had her left adrenal surgically removed by laparoscopy.

One week, 4 months, and 16 months after surgery the patient was again admitted to our unit for further evaluation.

Investigation protocol

The patient was studied according to a protocol that had been approved by the local ethical committee and by the patient, with a written informed consent. Studies were performed after an overnight fast, with the patient in the supine position for at least 60 min before testing. The protocol to screen for potential illicit adrenal receptors included serial measurement of cortisol and other hormones at different intervals during various tests, which were performed sequentially over the course of several days and according to the protocol suggested by Lacroix *et al.* (4). A change in plasma cortisol less than 25% from baseline (100%) was considered as no response, according to Mircescu *et al.* (6), a 25–49% change was considered a partial response, and a change of 50% or greater was considered a positive response. Tests included the administration of 100 μg GnRH iv (Relefact, Hoechst-Roussel, Montreal, Canada); 200 μg TRH iv (Relefact-TRH, Hoechst-Roussel); 2.5 μg desmopressin sc (Minirin, Ferring Pharmaceuticals Ltd., North York, Ontario, Canada); 10 IU arginin-vasopressin (AVP) im (Pitressin, Goldshield Pharmaceuticals, Croydon, UK); 1 mg glucagon iv (Eli Lilly, Indianapolis, IN); 10 mg cisapride orally (Prepulsid, Janssen Pharmaceuticals, Titusville, NJ); and 250 μg ACTH-(1–24) iv (Cortrosyn, Organon Inc., West Orange, NJ). Other tests included a standard mixed meal (517 kcal, 48% carbohydrates, 38% lipids, 14% proteins) and a posture test performed in a 2-h supine position followed by a 2-h ambulation period. A stimulation test using cisapride was repeated twice before and three times after surgery. ACTH stimulation test was performed before and after surgery.

Assays

Cortisol (plasma, urine, culture medium) was measured by an electrochemiluminescent assay (Roche Diagnostics, Monza, Italy). ACTH was measured by a chemiluminescent assay (Nichols Institute Diagnostics, San Clemente, CA).

Cell cultures

Adrenocortical fragments obtained at surgery were processed for cell preparation or frozen in liquid nitrogen and stored at −80 C. A large fragment was sent to the Pathology Department for histopathology that assessed the presence of a pattern of macronodular adrenal hyperplasia. Primary cell cultures were established within 1 h of surgery, as described by Munari-Silem *et al.* (14), with some modifications. Briefly, the tissue was freed of fat, minced, and incubated for 20 min at 37 C in PBS (Sigma Chemical Co., St Louis, MO) containing 2 mg/ml collagenase (Sigma Chemical Co.). To facilitate dispersion, tissue was minced with a Pasteur pipette with a fine heat-polished tip; the cell suspension was then filtered through a cell strainer (80-μm mesh, Sigma Chemical Co.) and centrifuged 10 min at 1400 rpm. The pellet was resuspended in a culture medium consisting of a 1:1 (vol/vol) mixture DMEM/F-12 with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, and enriched with a mixture of insulin/transferrin/selenium (Sigma Chemical Co.). Isolated cells were plated onto 35-mm diameter culture dishes at a density of 50–70 × 10^3 cells per dish and cultured at 37 C in 95% air/5% CO_2 in a fully humidified environment. Primary cultures were used within 3–4 d. The precise number of cells used in each experimental protocol was determined at the end of the experiments using a hemocytometer.

In vitro cortisol secretion studies

Cells were seeded in six-well plates. After 3–4 d of culture, the growth medium was removed, and the cells were accurately washed in PBS and incubated in phenol red- and serum-free medium containing 0.1% BSA. After 24 h, ACTH-(1–24) (1 nM), cisapride (1 μM), or a combination of ACTH-(1–24) (1 nM) and cisapride (1 μM) was added to the cultures. After 24 h, the medium was harvested and kept frozen at −20 C in aliquots (300–400 μl) until steroid hormone determination. Cells in phenol red- and serum-free medium containing 0.1% BSA were used as basal controls. After the 24-h harvesting, cells were trypsinized and counted using a hemocytometer. In the same experiment, each point was repeated in duplicate. The experiments were repeated twice. Cortisol secretion (picomoles per 10^6 cells) was expressed as percentage (mean ± SE) of the cortisol secretion in unstimulated cells (control).

RT-PCR for 5-HT$_4$, β1-adrenergic, and angiotensin II type 1 receptors

RT-PCR was performed on total RNA (1 μg for each reaction) extracted from a frozen tumoral fragment, according to the method described by Chomczynski and Sacchi (15). In addition, RNA extracted from normal adrenal glands, obtained during nephrectomy for renal carcinomas, normal human kidney, and myometrium, was used in RT-PCR experiments. A commercially available kit (SuperScript One Step RT-PCR System, Stratagene, La Jolla, CA) was used to prepare the mixture for RT-PCR. For the detection of 5-HT$_4$ receptor, mRNA primers and experimental conditions were as described previously by Lefebvre *et al.* (16). For the detection of angiotensin II type 1 (AT-1) receptors, specific primers were designed using the proprietary software Primer Express (Applied Biosystems Inc., Foster City, CA). The expression of β1-adrenergic receptors was investigated using specific primers, as described previously (17). The PCR products were subjected to agarose gel electrophoresis, blotted on a nylon membrane (in the case of 5-HT$_4$ receptor), and hybridized to a digoxigenin-labeled internal oligonucleotide (16). The hybridized cDNAs were detected by using an immunochemiluminescent method (Roche Diagnostics), as described previously (18). The primers and the probe were synthesized by MWG Biotech AG (Ebersberg, Germany). Additional RT-PCR was performed using primers specific for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, as described previously (19), to verify that the RNAs were not degraded.

Quantitative determination of 5-HT$_4$ receptor mRNA

Real-time RT-PCR based on TaqMan technologies for the determination of the levels of 5-HT$_4$ receptor mRNA was performed using the same primers and probe (labeled with 6-carboxy-fluorescein), as described by Cartier *et al.* (7), that recognize all 5-HT$_4$ receptor splice

variants. Then, 400 ng total RNA was reverse transcribed in 80 μl of final volume using the TaqMan Universal Master Mix (Applied Biosystems), following the manufacturer's instructions. The profile of the one-step RT reaction was 10 min at 25 C, 30 min at 48 C, and 5 min at 95 C. The cDNAs were then subjected to PCR, using the following conditions: 2 min at 50 C, 10 min at 95 C, followed by 40 cycles at 95 C for 15 sec and 60 C for 1 min in the ABI Prism 7700 Sequence Detector (Applied Biosystems). A calibration curve was generated using a single-stranded sense oligodeoxynucleotide spanning the sequence included between the primers, as described by Bustin (20). Serial 1:10 dilutions from 1 fg to 100 pg of this oligodeoxynucleotide were subjected to real-time RT-PCR. All the reactions were run in triplicates in the presence of no template controls. Because normalization to GAPDH as well as to other housekeeping genes has been shown not to be very accurate (20), the results were expressed as femtograms of 5-HT_4 receptor mRNA per microgram of total RNA (mean value of three determinations).

Characterization of 5-HT_4 receptor isoforms by RT-PCR

Characterization of 5-HT_4 receptor isoforms was performed as described previously, using primers specific for the different splice variants (7). The PCR products were subjected to agarose gel electrophoresis, blotted on nylon membranes, and hybridized to a digoxigenin-labeled internal oligonucleotide, which served as the probe. The sequence of the oligonucleotide was 5′-CCCTGGGCAGGTGTGGACTGC-3′ (Ref. 7). The hybridized cDNAs were detected by using an immunochemiluminescent method (Roche Diagnostics), as described previously (18). The primers and the probe were synthesized by MWG Biotech AG (Ebersberg, Germany).

Results

In vivo evaluation for the presence of illicit adrenal hormone receptors

There was no increase in plasma cortisol after GnRH (LH increased from 4.5 to 11.3 U/liter, and FSH increased from 13.3 to 19.2 U/liter), TRH [TSH increased from 1.07 (normal range, 0.35–3.5) to 8.58 mU/liter; and prolactin increased from 157.2 (normal range, 72–504) to 1085.8 mU/liter], desmopressin, AVP, glucagon, and meal test, whereas a partial response followed the upright posture (Table 1). Conversely, a marked increase, greater than the response observed after ACTH infusion (from 587 to 1843 nmol/liter, 314% of baseline value, considered as 100%) used as a reference test, was observed after cisapride administration (Table 1). Cisapride was administered twice before surgery, and the rise in plasma cortisol was almost superimposable (from 523 to 2658 and from 517 to 2866 nmol/liter, respectively) (Table 1). Baseline urinary concentration of 5-hydroxy indole acetic acid was within the normal range (4.73 mg/24 h; normal range, 2.0–6.0). One week after surgery, the patient was admitted to our unit for further evaluation. At that time she suffered from profound asthenia. Plasma ACTH was 10.5 ng/liter, and plasma cortisol was 94 nmol/liter at 0800 h and 92 nmol/liter at 2400 h. Urinary free cortisol was less than 25 nmol/24 h. Cisapride still caused an evident rise in plasma cortisol levels (from 107 to 664 nmol/liter, 620% of baseline value), and the increase was again greater than the response obtained after ACTH infusion (from 120 to 521 nmol/liter, 434% of baseline value). Cortisone acetate treatment was started (25 mg/d).

TABLE 1. Plasma cortisol responses to ACTH and to the tests performed to detect the presence of aberrant membrane hormone receptors

Test	Plasma cortisol at baseline (nmol/liter)	Peak plasma cortisol (nmol/liter)	Peak value as a percentage of baseline value (100%)
ACTH 250 μg	587	1843	314
GnRH 100 μg	567	541	95
TRH 200 μg	439	469	107
Desmopressin 2.5 μg	582	431	74
AVP 10 IU[a]	546	544	99
Glucagon 1 mg	579	522	90
Mixed meal	625	566	90
Upright posture	470	671	143
Cisapride 10 mg (first testing)	523	2658	508
Cisapride 10 mg (second testing)	517	2866	554

[a] Because plasma ACTH was not completely suppressed, 2 mg dexamethasone was administered orally the night before the test (2400 h) and the day of the test (0600 h). Plasma ACTH did not increase during the test.

Four months later the patient was again evaluated. She was still taking cortisone acetate, and her clinical conditions were markedly ameliorated since the last evaluation. After admission, cortisone supplementation was discontinued, and after 3 d basal hormonal assessment was performed. Plasma ACTH was 17.1 ng/liter, and plasma cortisol was 462 nmol/liter at 0800 h and 112 nmol/liter at 2400 h. Cisapride was once again administered, and plasma cortisol increased from 482 to 828 nmol/liter (172% of baseline value). Sixteen months after surgery, cisapride testing was again performed. Basal ACTH and cortisol plasma levels were 15.5 ng/liter and 360 nmol/liter, respectively, at 0800 h. Cisapride increased cortisol plasma level from 360 to 870 nmol/liter (241% of baseline value). Figure 1 details the cortisol response to cisapride administration before (*circles*) and after (*squares* and *triangles*) surgery.

In vitro cortisol secretion studies

Primary cell cultures were established within 1 h of surgery, as described in *Patient and Methods*. Treatment with

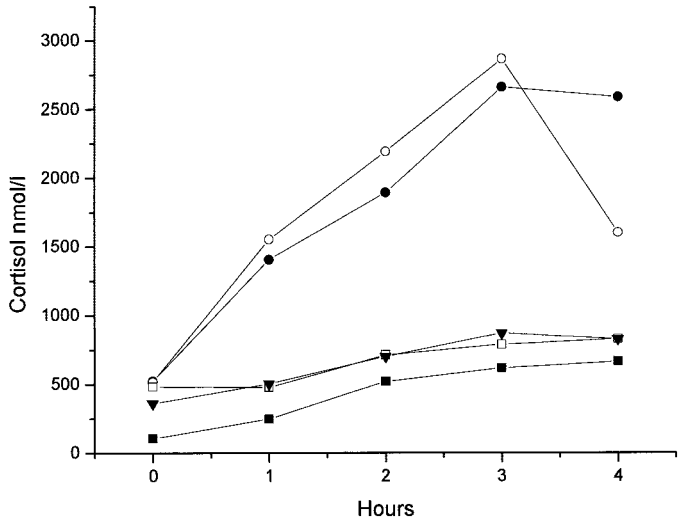

FIG. 1. Cortisol response to cisapride administration (10 mg orally) before surgery (*filled circles*, first testing; *open circles*, second testing) and after surgery (*filled squares*, 1 wk after surgery; *open squares*, 4 months after surgery; *filled triangles*, 16 months after surgery).

ACTH-(1–24) (1 nM), cisapride (1 μM), or a combination of ACTH-(1–24) (1 nM) and cisapride (1 μM) was performed, and after 24 h the medium was harvested for subsequent cortisol determination. Cortisol secretion (picomoles per 10^6 cells) in the medium was expressed as percentage (mean ± SE) of the cortisol secretion in unstimulated cells (control, 100%). The results, shown in Fig. 2, indicate that ACTH-(1–24) and cisapride, similar to *in vivo* observations, significantly increased cortisol secretion (171.5 ± 10.3% and 133.7 ± 5.5%, respectively), compared with unstimulated cells. The simultaneous treatment with ACTH-(1–24) and cisapride determined a dramatic increase of cortisol secretion (479.6 ± 68.4%) that was significantly greater than the increase observed with ACTH-(1–24) or cisapride alone. The number of cells ($\times 10^4$) per well did not significantly differ in the treated groups compared with the control group (26.15 ± 3.06, control; 25.68 ± 3.80, ACTH; 24.25 ± 0.75, cisapride; 21.37 ± 1.87, ACTH + cisapride; mean ± SE).

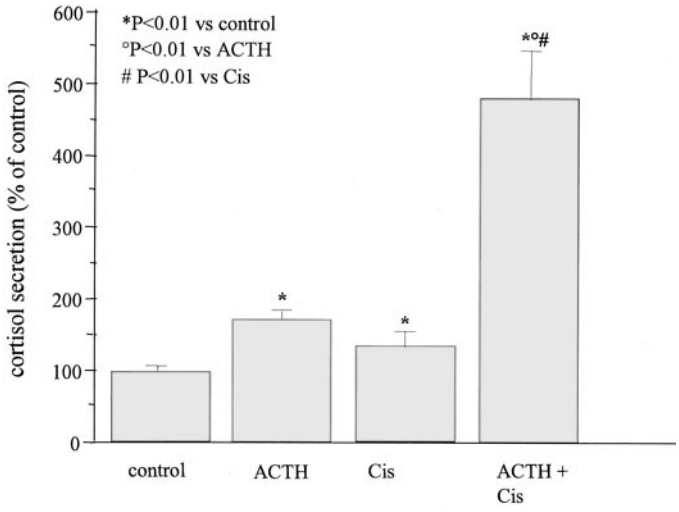

FIG. 2. *In vitro* response to ACTH (1 nM) and/or cisapride (Cis 1 μM), in terms of cortisol secretion.

Analysis of 5-HT$_4$, β1-adrenergic, and AT-1 receptor transcripts

The presence of the transcript corresponding to 5-HT$_4$ receptor was investigated by RT-PCR, using specific primers (see *Patient and Methods*). Total RNA extracted from the excised adrenal gland of the patient was used. In addition, RNA extracted from a normal adrenal gland, obtained during surgery for a renal carcinoma, was subjected to RT-PCR. RNA contamination was excluded by adding a "no RNA" control reaction to the experiment. The expected 346-bp signal was readily detectable after agarose gel electrophoresis from both the tumoral and the normal adrenal gland (Fig. 3A). The identity of 5-HT$_4$ receptor was confirmed by hybridization with a specific probe, which is derived from an entirely different region of the 5-HT$_4$ receptor cDNA from the primers (16) (Fig. 3B). In Fig. 3C, the signals corresponding to GAPDH transcript are shown. Because a partial response, in terms of plasma cortisol increase (143% of baseline value, considered as 100%; see Table 1), followed the upright posture, RT-PCR for the detection of AT-1 and β1-adrenergic receptors was also performed. Specific signals for both AT-1 (251 bp) and β1-adrenergic (227 bp) receptors were detected from the tumoral as well as from the normal adrenal gland RNA. RNA extracted from a normal kidney served as the positive control for AT-1 receptor, whereas RNA from human myometrium was the positive control for β1-adrenergic receptor (Fig. 4, A and B).

Quantitative determination of 5-HT$_4$ receptor mRNA

The quantitative expression of 5-HT$_4$ receptor mRNA was determined from total RNA extracted from the excised adrenal gland of the patient by real-time RT-PCR based on TaqMan technologies (see *Patient and Methods*) and com-

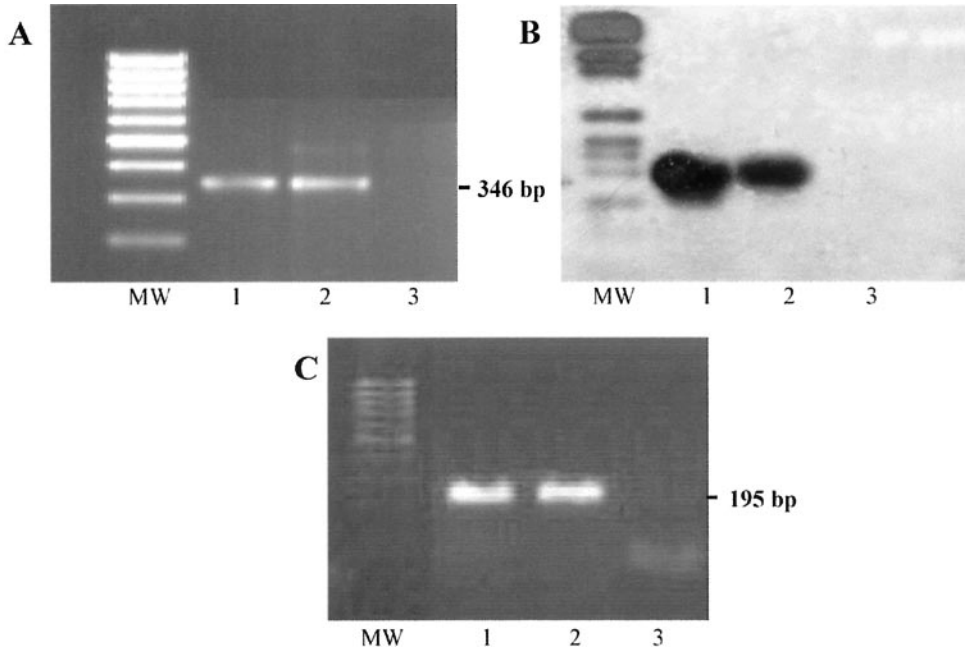

FIG. 3. A, Ethidium bromide-stained gel, showing 5-HT$_4$ receptor-specific RT-PCR products: MW, molecular weight marker VI (Roche Diagnostics); 1, tumoral adrenal sample; 2, normal adrenal gland; 3, no RNA control reaction. B, Hybridization pattern of RT-PCR products from the same experiment as A. C, Ethidium bromide-stained gel, showing GAPDH-specific RT-PCR products.

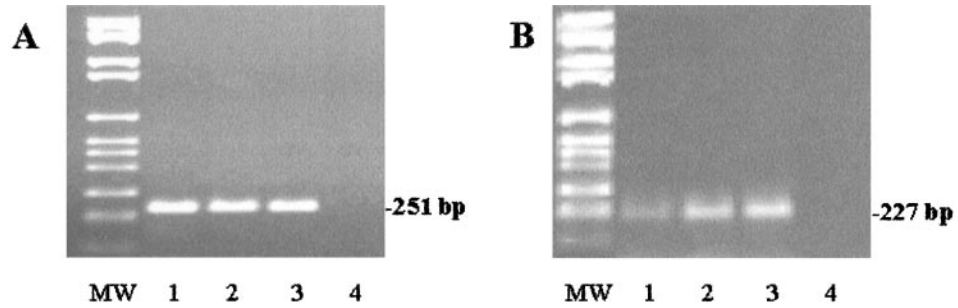

FIG. 4. Ethidium bromide-stained gels, showing AT-1 (A) and β-1 adrenergic (B) receptor RT-PCR products: MW, molecular weight marker VI (Roche Diagnostics); 1, tumoral adrenal sample; 2, normal adrenal gland; 3, human kidney (A) and human myometrium (B); and 4, no RNA control reaction.

TABLE 2. Expression levels of $5HT_4$ receptor mRNA in the tumoral adrenal gland (no. 1) and in five normal adrenal cortices (no. 2–6), determined by quantitative real-time RT-PCR

	$5HT_4$ receptor mRNA fg/μg total RNA
1	1.55
2	0.47
3	0.76
4	0.47
5	0.23
6	1.46

TABLE 3. RT-PCR analysis of $5HT_4$ receptor isoform (a–h, n) mRNA in the tumoral adrenal gland (no. 1) and in five normal adrenal cortices (no. 2–6)

	a	b	c	d	e	f	g	h	n
1	−	−	+	−	−	−	+	−	+
2	+	−	+	+	−	−	+	−	+
3	+	−	+	+	−	−	+	−	+
4	+	−	+	−	−	−	+	−	+
5	+	−	+	−	−	−	+	−	+
6	+	−	+	+	−	−	+	−	+

pared with the expression levels of five normal adrenal glands, obtained during nephrectomy for renal carcinomas. The results are shown in Table 2 and indicate that the highest level of 5-HT_4 receptor mRNA (1.55 fg/μg total RNA) was found in the tumoral gland (no. 1). The mean value of the normal adrenal glands was 0.68 fg/μg total RNA, and in only one case (no. 6) was the level of expression above 1 fg/μg total RNA.

Characterization of 5-HT_4 receptor isoforms by RT-PCR

5-HT_4 receptor isoforms were characterized by RT-PCR in the tumoral adrenal gland and in the five normal adrenal glands. In the tumoral tissue, isoforms c, g, and n could be detected (Table 3). With regard to the normal adrenal glands, isoforms a, c, g, and n were detected in all cases and isoform d was present in three cases, whereas isoforms b, e, f, and h were not detectable in any case.

Discussion

In this manuscript, we reported the case of a patient affected by Cushing's syndrome due to AIMAH, in which the 5-HT_4 receptor, among the tested illicit adrenal receptors (4), dramatically regulated cortisol secretion. Aberrant serotonin receptors have been described in patients with adrenal Cushing's syndrome, in association with other aberrant receptors such as LH/hCG, V_1-vasopressin, or β-adrenergic receptors (5–8). In only one case, recently described, cortisol secretion appeared to be markedly modulated exclusively by 5-HT_4 receptors (7). In our patient a slight increase, defined as a partial response (6), in serum cortisol was observed after upright posture (43%), and the presence of β1-adrenergic and AT-1 receptors mRNA was detected by RT-PCR. However, the clinical significance of this finding appears to be minor because the effect of posture was absent every time the circadian rhythm of cortisol secretion was evaluated. On the contrary, in our patient a marked response to cisapride was observed and constantly found in different occasions before surgery. The presence of aberrant receptors on cortisol secreting adenomas does not prove by itself that they are responsible for Cushing's syndrome. Only when chronic hypercortisolism is reverted by the administration of specific receptor antagonists can the pathogenetic role of aberrant receptors be proved (5, 9). Because no specific antagonist of 5-HT_4 receptors is available for *in vivo* use, the role played by endogenous serotonin on the genesis of Cushing's syndrome in our patient, as well as in the other similar cases described so far (5–8), remains controversial. The patient's urinary 5-hydroxy indole acetic acid levels were within the normal range, but it is well known that in human adrenocortical cells serotonin, locally released by mast-cells, may stimulate both cortisol and aldosterone secretion in a paracrine manner (21–25). In our patient, the finding of hypocortisolism after the removal of the largest adrenal gland, in association with a persistent response to cisapride by the contralateral gland, suggests that the clinical picture might depend on the presence of a critically enlarged mass, with a high number of secreting cells, expressing 5-HT_4 receptors. It has to be said that 5-HT_4 receptors have been detected also in the normal adrenal cortex (21–25). However, in that case they were found to be mainly linked to aldosterone secretion. Furthermore, as suggested by Lacroix *et al.* (4), the response to cisapride in patients with Cushing's syndrome might be due to increased expression or abnormal function of eutopic 5-HT_4 receptors in the adrenal cortex. With regard to this hypothesis, quantitative determination of the level of 5-HT_4 receptor mRNA by real-time RT-PCR indicated higher expression in the tumoral adrenal gland from our patient with AIMAH than in normal adrenal glands. Accordingly, overexpression of 5-HT_4 receptors in the adrenal cortex of four of six patients with AIMAH, compared with the normal adrenal gland, has been very recently described (7). In that study, additional characterization of 5-HT_4 receptor isoforms (10–13) revealed that in the two cases in which overexpression of

5-HT_4 receptor did not occur, variants present also in normal glands (a–d and f) were found. Furthermore, no gain-of-function mutations of the receptor were detected, leaving the dilemma regarding the pathogenic role of aberrant adrenal receptors at least partially unsolved so far. In the adrenal gland excised from our patient, isoforms c, g, and n were detected. These isoforms were detectable also in the normal adrenal glands included in our study. However, in normal glands, isoform a was also present in all cases, and isoform d was detectable in three cases. Therefore, it might be hypothesized that increased expression of eutopic receptors and/or the presence of particular isoform clusters play a role in determining abnormal responses elicited by *in vivo* testing at least in a subset of adrenal tumors expressing 5-HT_4 receptors. Although in normal subjects 5-HT_4 receptor agonists do not increase plasma cortisol levels (24), the persistence of the response to cisapride in our patient after left adrenalectomy may be due to the presence of an enlarged, yet not hyperfunctioning, gland. The possibility that in the adrenal cortex a better coupling of illicit receptors to proliferative signals than to hormone synthesis occurs has been suggested recently in the paper by Bourdeau *et al.* (8).

Overall, although the cause of Cushing's syndrome in the case we described remains speculative, it is of interest that in *in vitro* experiments cisapride greatly augmented ACTH-induced cortisol secretion. In our patient, plasma ACTH was still measurable, as reported in other patients with AIMAH (8), and hypercortisolism might be caused by a combined stimulus on a highly enlarged population of adrenal cells.

The reversal of the clinical picture that we observed in our patient after unilateral adrenalectomy has already been reported in the literature in patients with AIMAH (26). In agreement with the experience of other authors, in our patient the unilateral removal of the largest adrenal gland caused, as above mentioned, a dramatic decrease of cortisol production and improved the clinical picture. Hypocortisolism, which had to be corrected by oral cortisone supplementation, ensued soon after surgery and lasted for several months. At present, plasma and urinary cortisol are normal, and the patient is doing well despite the contralateral enlarged adrenal gland. Therefore, we confirm that unilateral adrenalectomy may be a safe and long-lasting therapy in patients with Cushing's syndrome due to AIMAH. Whether the remaining adrenal gland will enlarge and cause a recurrence of hypercortisolism in the future is unknown, but until then a condition of permanent Addison's disease will be avoided.

In conclusion, in the present study we reported the case of a patient with Cushing's syndrome due to AIMAH, in which cortisol secretion was markedly stimulated by cisapride. Interestingly, the *in vivo* response was maintained after removal of the largest adrenal gland, also during a state of transient hypocortisolism. *In vitro* studies confirmed that the response to cisapride was due to the presence of 5-HT_4 receptors and indicated that the expression level was higher than in normal adrenal glands. At present, the role played by illicit receptors in the pathogenesis of Cushing's syndrome is controversial. Nevertheless, the presence of illicit receptors such as 5-HT_4 receptors in the adrenal glands of patients with hypercortisolism should be investigated, because the eventual availability of specific blockers of 5-HT_4 receptors could be of interest for the potential treatment of such patients.

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References

1. **Latronico AC, Chrousos GP** 1997 Extensive personal experience: adrenocortical tumors. J Clin Endocrinol Metab 82:1317–1324
2. **Bornstein SR, Stratakis CA, Chrousos GP** 1999 Adrenocortical tumors: recent advances in basic concepts and clinical management. Ann Intern Med 130: 759–771
3. **Kirschner LS, Carney A, Pack SD, Taymans S, Giatzakis C, Cho YS, Cho-Chung YS, Stratakis CA** 2000 Mutations of the gene encoding the protein kinase A type I-α regulatory subunit in patients with the Carney complex. Nat Genet 26:89–92
4. **Lacroix A, N'Diaye N, Tremblay, Hamet P** 2001 Ectopic and abnormal hormone receptors in adrenal Cushing's syndrome. Endocr Rev 22:75–110
5. **Lacroix A, Hamet P, Boutin J-M** 1999 Leuprolide acetate therapy in luteinizing hormone-dependent Cushing's syndrome. N Engl J Med 341:1577–1581
6. **Mircescu H, Jilwan J, N'Diaye N, Bourdeau I, Tremblay J, Hamet P, Lacroix A** 2000 Are ectopic or abnormal membrane hormone receptors frequently present in adrenal Cushing's syndrome? J Clin Endocrinol Metab 85:3531–3536
7. **Cartier D, Lihrmann I, Parmentier F, Bastard C, Bertherat J, Caron P, Kuhn J-M, Lacroix A, Tabarin A, Young J, Vaudry H, Lefebvre H** 2003 Overexpression of serotonin$_4$ receptors in cisapride-responsive adrenocorticotropin-independent bilateral macronodular hyperplasia causing Cushing's syndrome. J Clin Endocrinol Metab 88:248–254
8. **Bourdeau I, D'Amour P, Hamet P, Boutin J-M, Lacroix A** 2001 Aberrant membrane hormone receptors in incidentally discovered bilateral macronodular adrenal hyperplasia with subclinical Cushing's syndrome. J Clin Endocrinol Metab 86:5534–5540
9. **Lacroix A, Tremblay J, Rousseau G, Bouvier M, Hamet P** 1997 Propranolol therapy for ectopic β-adrenergic receptors in adrenal Cushing's syndrome. N Engl J Med 337:1429–1434
10. **Blondel O, Vandecasteele G, Gastineau M, Leclerc S, Dahmoune Y, Langlois M, Fischmeister R** 1997 Molecular and functional characterization of a 5-HT_4 receptor cloned from human atrium. FEBS Lett 412:465–474
11. **Blondel O, Gastineau M, Dahmoune Y, Langlois M, Fischmeister R** 1998 Cloning, expression, and pharmacology of four human 5-hydroxytryptamine 4 receptor isoforms produced by alternative splicing in the carboxyl terminus. J Neurochem 70:2252–2261
12. **Vilaro MT, Domenech T, Palacios JM, Mengod G** 2002 Cloning and characterization of a novel human 5-HT_4 receptor variant that lacks the alternatively spliced carboxy terminal exon. RT-PCR distribution in human brain and periphery of multiple 5-HT_4 receptor variants. Neuropharmacology 42:60–73
13. **Bender E, Pindon A, Van Oers I, Zhang YB, Gommeren W, Verhasselt P, Jurzak M, Leysen J, Luyten W** 2000 Structure of the human serotonin 5-HT_4 receptor gene and cloning of a novel 5-HT_4 splice variant. J Neurochem 74:478–489
14. **Munari-Silem Y, Lebrethon MC, Morand I, Rousset B, Saez JM** 1995 Gap junction-mediated cell-to-cell communication in bovine and human adrenal cells. A process whereby cells increase their responsiveness to physiological corticotropin concentrations. J Clin Invest 95:1429–1439
15. **Chomczynski P, Sacchi N** 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
16. **Lefebvre H, Cartier D, Duparc C, Lihrman I, Contesse V, Delarue C, Godin M, Fischmeister R, Vaudry H, Kuhn J-M** 2002 Characterization of serotonin$_4$ receptors in adrenocortical aldosterone-producing adenomas: *in vivo* and *in vitro* studies. J Clin Endocrinol Metab 87:1211–1216
17. **Bahouth SW, Sowinski KM, Lima JJ** 2001 Regulation of human β_1-adrenergic receptors and their mRNA in neuroepithelioma SK-N-MC cells: effects of agonist, forskolin, and protein kinase A. Biochem Pharmacol 62:1211–1220
18. **Peri A, Cordella-Miele E, Miele L, Mukherjee AB** 1993 Tissue-specific expression of the gene coding for human Clara cell 10-kD protein, a phospholipase A_2-inhibitory protein. J Clin Invest 92:2099–2109
19. **Dveksler GS, Basile AA, Dieffenbach CW** 1992 Analysis of gene expression: use of oligonucleotide primers for glyceraldehyde-3-phosphate dehydrogenase. PCR Methods Applic 1:283–285

20. **Bustin SA** 2002 Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 29:23–39
21. **Lefebvre H, Contesse, Delarue C, Feuilloley M, Hery F, Grise P, Raynaud G, Verhofstad AA, Wolf LM, Vaudry H** 1992 Serotonin-induced stimulation of cortisol secretion from human adrenocortical tissue is mediated through activation of a serotonin 4 receptor subtype. Neuroscience 47:999–1007
22. **Lefebvre H, Contesse V, Delarue C, Soubrane C, Legrand A, Kuhn JM, Wolf LM, Vaudry H** 1993 Effect of the serotonin-4 receptor agonist zacopride on aldosterone secretion from the human adrenal cortex. In vivo and *in vitro* studies. J Clin Endocrinol Metab 77:1662–1666
23. **Lefebvre H, Contesse V, Delarue C, Legrand A, Kuhn JM, Vaydry H, Wolf LM** 1995 The serotonin-4 receptor agonist cisapride and angiotensin-II exert additive effects on aldosterone secretion in normal man. J Clin Endocrinol Metab 80:504–507
24. **Lefebvre H, Contesse V, Delarue C, Vaudry H, Kuhn JM** 1998 Serotoninergic regulation of adrenocortical function. Horm Metab Res 30:398–403
25. **Lefebvre H, Compagnon P, Contesse V, Delarue C, Thuillez C, Vaudry H, Kuhn JM** 2001 Production and metabolism of serotonin (5-HAT) by the human adrenal cortex: paracrine stimulation of aldosterone secretion by 5-HAT. J Clin Endocrinol Metab 86:5001–5007
26. **Lamas C, Alfaro JJ, Lucas T, Lecumberri B, Barcelò, Estrada J** 2002 Is unilateral adrenalectomy an alternative treatment for ACTH-independent macronodular adrenal hyperplasia?: long-term follow-up of four cases. Eur J Endocrinol 146:237–240