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In Vivo and *in Vitro* Screening for Illegitimate Receptors in Adrenocorticotropin-Independent Macronodular Adrenal Hyperplasia Causing Cushing's Syndrome: Identification of Two Cases of Gonadotropin/Gastric Inhibitory Polypeptide-Dependent Hypercortisolism

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In ACTH-independent macronodular adrenal hyperplasia (AIMAH) causing Cushing's syndrome, cortisol production can be controlled by illegitimate membrane receptors. The aim of the present study was to evaluate *in vivo* and *in vitro* the sensitivity of AIMAH to various regulatory factors to detect the expression of illegitimate receptors by the tissues. Four consecutive patients with AIMAH and hypercortisolism (H1–H4) preoperatively underwent a series of pharmacological and/or physiological tests. After adrenalectomy, *in vitro* studies were conducted to investigate the cortisol responses of cultured cells, derived from hyperplastic tissues, to various membrane receptor ligands. The adrenal tissues of the two patients who responded *in vivo* to food intake (H2 and H4) were stimulated *in vitro* by gastric inhibitory polypeptide. GnRH and human chorionic gonadotropin, but not FSH, stimulated cortisol secretion in patients H2 and H4. In these two cases, human chorionic gonadotropin but not GnRH stimulated cortisol production from cultured adrenocortical cells. Cisapride induced a significant increase in cortisol levels in patient H1. In addition, serotonin (5-HT) was more efficient to stimulate cortisol production in H1 cells than in normal adrenocortical cells. Upright stimulation test provoked an increase in cortisol levels in patients H1, H2, and H3. H1 and H2 cells were more sensitive to the stimulatory action of angiotensin II than normal cells. Similarly, arginine vasopressin (AVP) more efficiently activated steroidogenesis in H1 cells than in normal cells. In H1 tissue, immunohistochemical studies revealed the presence of 5-HT- and AVP-like immunoreactivities within clusters of steroidogenic cells, suggesting that these two factors acted through an autocrine/paracrine mechanism to stimulate cortisol secretion. The present study provides the first demonstration of primary adrenal Cushing's syndrome dependent on both gonadotropin and gastric inhibitory polypeptide. Our data also show a hyperresponsiveness of hyperplastic adrenal tissues to 5-HT, angiotensin II, and AVP. Finally, they reveal for the first time the presence of paracrine regulatory signals in adrenal hyperplasia tissues. (*J Clin Endocrinol Metab* 90: 1302–1310, 2005)

ILLEGITIMATE MEMBRANE RECEPTORS have been shown to be involved in cortisol production in both adrenal adenomas and ACTH-independent macronodular adrenal hyperplasia (AIMAH) causing Cushing's syndrome (1). These aberrant receptors include ectopic receptors for gastric inhibitory polypeptide (GIP), LH, or catecholamines, and abnormally active eutopic receptors like vasopressin V_{1a} and serotonin$_4$ (5-HT$_4$) receptors (1). The current approach used for the detection of illegitimate receptors consists of measuring plasma cortisol levels (PCL) in response to various physiological and pharmacological tests comprising a posture test, a standard mixed meal, parenteral administration of GnRH and arginine vasopressin (AVP), and oral administration of the 5-HT$_4$ receptor agonists cisapride and metoclopramide (2). This screening protocol has revealed that the presence of illegitimate receptors is frequent in AIMAHs, opening new perspectives for the development of medical therapies in adrenal Cushing's syndrome (3, 4). However, the diverse regulatory mechanisms involved in the pathophysiology of the disease, especially in the maintenance of cortisol hypersecretion in the absence of circulating ACTH, cannot be identified by the sole use of clinical testing for several reasons. First, the sensitivity of pharmacological tests is necessarily limited by the bioavailability of the test molecules. Second, the interpretation of a positive cortisol response to a physiological stimulation test, like food intake or upright stimulation test, is often difficult because the ad-

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Abbreviations: AIMAH, ACTH-independent macronodular adrenal hyperplasia; Ang II, angiotensin II; AVP, arginine vasopressin; CT, computerized tomography; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; hCG, human chorionic gonadotropin; 5-HT, serotonin; PCL, plasma cortisol level(s).

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renal secretory response may involve several hormonal signals (1). For instance, the increase in cortisol levels evoked by a mixed meal can be the result of an abnormal sensitivity of the adrenal tissue to either GIP or other gastrointestinal hormones like glucagon-like peptide-1 (GLP-1). Third, a pharmacological agent may stimulate cortisol secretion through either a direct action on adrenocortical cells or an indirect effect on other intraadrenal cell types that, in turn, may modulate the synthesis of glucocorticoids through a cell-to-cell mode of communication. In this regard, it has been reported that AVP is able to trigger the secretion of bioactive signals by human adrenomedullary cells, which then influence adrenocortical function (5, 6). *In vitro* studies are therefore required to detect abnormal sensitivity of adrenocortical cells to various stimuli in primary adrenal hypercortisolism.

The presence of ectopic GIP receptors in food-dependent adrenal lesions causing Cushing's syndrome has been demonstrated by *in vitro* incubation, *in situ* hybridization, and RT-PCR studies (7–10). Similarly, overexpression of the mRNAs encoding the eutopic V_{1a} and 5-HT_4 receptors has been described recently in tissue explants removed from AIMAHs responsive *in vivo* to AVP and cisapride, respectively (11–14). The normal human adrenal cortex contains substantial amounts of AVP and 5-HT, which can enhance cortisol secretion through paracrine mechanisms (15–17). Thus, it is conceivable that these factors may stimulate cortisol secretion by AIMAHs via activation of the abnormally expressed V_{1a} and 5-HT_4 receptors. However, the occurrence of AVP and 5-HT in hyperplastic adrenal tissues and the sensitivity of AIMAH cells to AVP and 5-HT, in comparison with normal adrenocortical cells, have never been investigated.

The aim of the present study was to evaluate *in vivo* and *in vitro* the responsiveness of AIMAH to various bioactive signals to detect the expression of illegitimate receptors by the tissues and, consecutively, determine the contribution of illegitimate receptors to the pathophysiology of the disease. For this purpose, four consecutive AIMAH patients with adrenal Cushing's syndrome systematically underwent a series of physiological and pharmacological tests before adrenalectomy. After surgery, *in vitro* studies were conducted to examine the effects of the regulatory factors that were potentially involved in the abnormal *in vivo* cortisol responses on glucocorticoid production by cultured cells derived from hyperplastic tissues, in comparison with normal adrenocortical cells. In addition, the possible occurrence of 5-HT and AVP within the hyperplastic tissues abnormally sensitive to these regulatory signals was investigated by immunohistochemistry.

Patients and Methods

Diagnosis of Cushing's syndrome

The diagnosis of ACTH-independent Cushing's syndrome was based on the results of hormonal investigations as reported previously (18–21). Briefly, an increase in 24-h urinary cortisol excretion, alteration of plasma cortisol circadian rhythm, lack of cortisol suppression under a low-dose dexamethasone test (2 mg/d for 2 d), and suppression of basal plasma ACTH levels [<5 pg/ml (1 pmol/liter)] were observed in the four patients.

Patients

Four consecutive patients with AIMAH responsible for Cushing's syndrome were investigated.

Patient 1, a 34-yr-old woman, was referred for diabetes mellitus, hypokalemia (3.4 mmol/liter), and hypertension (blood pressure, 180/100 mm Hg). She had hypertension during each of three full-term pregnancies, occurring respectively 6, 5, and 1.5 yr earlier. Gestational diabetes mellitus was also detected during the third pregnancy and treated with insulin. Physical examination revealed faciotruncal adiposity. Menstrual cycles were regular. ACTH-independent Cushing's syndrome was evidenced by hormonal investigations (Table 1). Abdominal computerized tomography (CT) showed multinodular enlargement of both adrenal glands that reached 4 cm in diameter on the right and 6 cm in diameter on the left.

Patient 2, a 45-yr-old woman, was referred for a recent history of hypokaliemia (3.06 mmol/liter) and hypertension (blood pressure, 160/120 mm Hg). She had a normal full-term pregnancy at the age of 27 yr. Several signs and symptoms including asthenia, depressive mood, facial erythrosis, faciotruncal adiposity, and mild facial hirsutism were suggestive of a mild Cushing's syndrome. Menstrual cycles had become irregular 4 months earlier. ACTH-independent Cushing's syndrome was diagnosed by routine hormonal investigations (Table 1). The analysis of circadian cortisol rhythm was highly suggestive of food-dependent Cushing's syndrome. Abdominal CT scan revealed multinodular enlargement of the two adrenal glands that reached 3.5 cm in diameter on the right and 4 cm in diameter on the left.

TABLE 1. Physical characteristics and basal hormone levels in the four patients with AIMAH

	Patient 1	Patient 2	Patient 3[a]	Patient 4	Normal range
Gender	F	F	F	F	
Age (yr)	34	45	49	54	
Body weight (kg)	78	60	68	60	
Urinary cortisol (μg/d)	135	525	118	707	20–90
Plasma cortisol 0800 h (μg/liter)	185	60	289	159	100–200
Plasma cortisol 2000 h (μg/liter)	169	28	221	338	
Plasma ACTH 0800 h (pg/ml)	<2	<5	<5	<5	20–60
Plasma aldosterone supine (pg/ml)	352	78	190	38	20–130
Plasma renin supine (pg/ml)	7	<1	191	23	5–40
Plasma testosterone (μg/liter)	<0.10	nd	0.10	0.70	0.20–0.80
Plasma DHEAS (μg/ml)	0.20	0.20	0.30	0.45	0.90–2.80
Plasma estradiol (pg/ml)	16	25	63	39	50–70
Plasma LH (U/liter)	0.8	1.5	22	12	1–13
Plasma FSH (U/liter)	1.6	4.4	16	32	1.5–12

F, Female; nd, not determined; DHEAS, dehydroepiandrosterone sulfate. Conversion factors are: cortisol (nanomoles, μg × 2.76), ACTH (picomoles per liter, pg/ml × 0.22), aldosterone (picomoles per liter, pg/ml × 2.77), renin (picomoles per liter, pg/ml × 0.0237), testosterone (nanomoles per liter, μg/liter × 3.47), DHEAS (micromoles per liter, μg/ml × 2.71), and estradiol (picomoles per liter, pg/ml × 3.67).

[a] The patient had received diuretics before hormone assays.

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Patient 3, a 49-yr-old woman, was referred for a recent disequilibrium of a 10-yr duration diabetes mellitus. She had two normal pregnancies at the ages of 23 and 25 yr. She also had a history of hypertension for 10 yr and heart failure. She presented with proximal myopathy, large ecchymotic lesions, facial erythrosis, hirsutism, faciotruncal adiposity, and spaniomenorrhea. ACTH-independent Cushing's syndrome was demonstrated by hormonal investigations (Table 1). Abdominal CT scan showed a multinodular enlargement of both adrenal glands that reached 5 cm in diameter on the right and 5.5 cm in diameter on the left.

Patient 4, a 54-yr-old woman, was referred for clinical signs of Cushing's syndrome including large ecchymotic lesions and central obesity, with a 12-kg weight gain during the last year. She had hypertension, which was treated for 18 months, and a 2.5-yr history of unexplained increase in circulating neutrophils. Physical examination revealed mild facial hirsutism, proximal myopathy, and skin thinning. She had undergone hysterectomy without oophorectomy at the age of 42 because of uterine fibromas. She had two normal pregnancies at the ages of 29 and 31 and an extrauterine pregnancy at the age of 39. ACTH-independent Cushing's syndrome was evidenced by hormonal investigations (Table 1). The analysis of circadian cortisol rhythm was highly suggestive of food-dependent Cushing's syndrome. Abdominal CT scan revealed bilateral macronodular adrenal hyperplasia that reached 5 cm in diameter on the right and 5.4 cm in diameter on the left.

All patients underwent bilateral laparoscopic adrenalectomy, and macronodular adrenocortical hyperplasia was confirmed by pathological examination.

Clinical investigation protocol

Potentially illegitimate membrane hormone receptors were systematically searched for, after informed consent of the patients, by using a modified version of the clinical protocol previously published by Lacroix *et al.* (1). The study was approved by the institutional ethics committee. It was performed on 4 consecutive days after the initial routine hormonal investigations. Briefly, PCL were measured in response to a posture test, a standard mixed meal, administration of 250 μg $ACTH_{1-24}$ iv used as a reference test, combined iv administration of 100 μg GnRH and 250 μg TRH, 10 mg cisapride (Prépulsid, Janssen-Cilag Laboratories, Boulogne-Billancourt, France) orally or 10 mg metoclopramide (Primpéran, Synthélabo Laboratories, Meudon-la-Forêt, France) iv, 1 mg glucagon im, and 0.5 mg terlipressin (a precursor of lysine vasopressin; Glypressine, Ferring Laboratories, Gentilly, France) iv. In patients 2 and 4, TRH and GnRH tests were repeated separately to investigate the cortisol response to the combined TRH/GnRH stimulation test. Patients 2 and 4 also underwent human chorionic gonadotropin (hCG) (5000 IU im) (Gonadotrophine Chorionique Endo, Organon, Eragny-sur-Epte, France) and recombinant human FSH (150 IU im) (Gonal-f, Serono, Boulogne, France) stimulation tests. The four patients studied were maintained in a recumbent position during all pharmacological tests. Plasma cortisol and ACTH assays were performed as described previously (18–21). In accordance with previous reports (2–4), a cortisol response was considered nonsignificant when less than 25%, potentially significant when above 25%, and highly significant when above 50% of basal level.

In vitro studies

Tissue collection. Adrenocortical tissues were obtained at surgery and immediately dissected by the pathologist. Normal adrenal explants (control tissues) were obtained from patients undergoing expanded nephrectomy for kidney cancer. Adrenocortical fragments were either transported to the laboratory in culture medium for primary culture and perifusion experiments or fixed in formalin and embedded in paraffin for immunohistochemical analysis. The protocol of collection of the tissues and the experimental procedures were approved by the regional ethics committees, and written informed consent was obtained from all subjects.

Reagents. Protease, collagenase (type IA), deoxyribonuclease I, insulin, apo-transferrin, L-ascorbic acid, 5-HT, isoproterenol, AVP, angiotensin II (Ang II), and the rabbit 5-HT antiserum were purchased from Sigma (St-Quentin Fallavier, France). The nutrient medium F-12 (Ham's) and DMEM were obtained from Life Technologies, Inc. (Paisley, Scotland, UK). The antibiotic-antimycotic solution and fetal bovine serum were from Bio-Whittaker (Walkersville, MD). GIP and GLP-1-(7–36) amide were from Bachem (Budendorf, Switzerland). GnRH and ACTH were from Ferring SAS (Gentilly, France). hCG was from Organon, and recombinant human FSH was from Serono.

Cell culture. Hyperplasia and normal adrenal gland fragments were immersed in culture medium (50% DMEM to 50% Ham's F-12) supplemented with 0.2% antibiotic-antimycotic solution and rapidly transported to the laboratory. The adrenal cortex was dissected from fat and medullary tissues and minced with scissors, and adrenal cells were enzymatically dispersed, as described previously (22). Briefly, tissue samples were stirred for 45 min at 37 C in culture medium containing protease (2 mg/ml), collagenase (2 mg/ml), and desoxyribonuclease I (70 μg/ml) in a 5% CO_2-95% air atmosphere. The tissues were disaggregated by gentle aspirations with a sterile 10-ml pipette. Dispersed cells were filtered on a nylon sieve (100-μm mesh opening). The cell suspension was centrifuged (100 $\times$ *g*, 37 C, 20 min). Residual tissue fragments were subjected to a second period of digestion/dispersion procedure as described above. Isolated cells were then transferred into culture medium supplemented with 5 μg/ml insulin, 10 μg/ml apotransferrin, 20 mg/ml ascorbic acid, and 5% fetal calf serum. Adrenocortical cells were cultured in petri dishes (at a density of 10^6 cells per dish) and incubated at 37 C in a 5% CO_2-95% air atmosphere with 100% relative humidity. The culture medium was changed 24 h after plating. Incubation experiments of cells with 5-HT were conducted after 2 d in culture with fresh DMEM (control) or DMEM containing different concentrations of GIP, GLP-1, hCG, GnRH, AVP, 5-HT, ACTH, Ang II, or isoproterenol. Cells were incubated with each secretagogue for 24 h at 37 C. After the incubation period, aliquots of the culture medium were taken and immediately frozen at −20 C until cortisol RIA. Results are expressed as mean ± SE, and statistical significance was assessed by Bonferroni test after one-way ANOVA.

Perifusion experiments. Hyperplasia tissues obtained at surgery from patients 2 and 4 were studied by use of a perifusion system technique as described previously (16). Briefly, hyperplasia fragments were dipped into 100 ml DMEM and rapidly transported to the laboratory. The adrenocortical explants were diced into small pieces (1–2 mm^3), rinsed three times with fresh medium, mixed with biogel P2, and transferred into perifusion chambers. The tissue fragments were perifused with DMEM at constant flow rate (300 μl/min), pH (7.4), and temperature (37 C). The perifusion medium was continuously gassed with a 95% O_2-5% CO_2 mixture. The tissues were allowed to stabilize for 2 h before any test substance was administered. Test substances were dissolved in gassed DMEM and infused into the perifusion chambers at the same flow rate as DMEM alone, by means of a multichannel peristaltic pump. Fractions of the effluent perifusate were collected every 5 min and immediately frozen until assay. Cortisol levels were determined in all fractions by RIA, as described previously (16).

Immunohistochemistry. Deparaffinized sections from hyperplasia tissues were incubated overnight at 4 C in a humidified atmosphere, with polyclonal rabbit antibodies directed against either 5-HT (1:400) or AVP (1:400) (provided by Dr. A. Burlet, Nancy University, Nancy, France). The sections were then incubated with a streptavidin-biotin-peroxidase complex (Dako Corporation, Carpinteria, CA), and the enzymatic activity was revealed with 3-amino-ethylcarbazole. The specificities of the immunoreactions were controlled by using 5-HT- or AVP-antisera preabsorbed for 2 h at 20 C with 10^{-6} M 5-HT or 10^{-6} M AVP, respectively. The tissue sections were counterstained for 1 min with hematoxylin, mounted in Eukitt (Kindler Gmbh & Co., Freiburg, Germany), coverslipped, and examined on an Eclipse E600 microscope (Nikon, Les Ulis, France) equipped with a charge-coupled device DXC-950 camera (Sony, Paris, France).

Results

In all four patients, plasma ACTH levels remained suppressed [5 pg/ml (1 pmol/liter)] throughout the *in vivo* studies (data not shown), indicating that all of the plasma cortisol responses observed were not mediated by ACTH.

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Effect of food intake on cortisol secretion

In patients 2 and 4, the mixed meals induced a robust increase in plasma cortisol (Table 2 and Fig. 1A). The plasma circadian cortisol rhythm was suggestive of food-dependent Cushing's syndrome in both cases (data not shown), although in patient 4, fasting PCL remained relatively high (Table 1). In contrast, mixed meals had no influence on plasma cortisol in patients 1 and 3. Incubation of cultured adrenocortical cells obtained from patients 2 and 4 with increasing concentrations of GIP (10^{-10} to 10^{-6} M) provoked a dose-dependent stimulation of cortisol secretion, whereas administration of GLP-1 (10^{-10} to 10^{-6} M) had no effect (Fig. 1B). GIP was equally potent to stimulate steroidogenesis from patient 2 hyperplasia (H2) and patient 4 hyperplasia (H4) cells (pEC_{50} were 9.6 ± 0.3 and 10.2 ± 0.2, respectively; mean ± SE, n = 4), but was more efficient to stimulate cortisol production from H2 cells (E_{max} = +320 ± 31%; n = 4) than H4 cells (E_{max} = +211 ± 8%; n = 4; $P < 0.01$). The effect of GIP (10^{-7} M) on cortisol secretion was also evaluated in adrenocortical cell cultures derived from patients 1 and 3 who did not respond to food intake. Surprisingly, GIP induced a +106% increase in cortisol secretion from H1 cells, whereas it had no affect on H3 cells. Administration of GIP (10^{-7} M; 20 min) to perifused H2 explants induced a massive but transient increase in cortisol release that reached a maximum of +854 ± 189% (n = 3; Fig. 1C).

Effects of GnRH and hCG on cortisol secretion

A significant increase in PCL was observed in patients 2 and 4 (+463 and +194%, respectively) after combined iv administration of TRH (250 μg) and GnRH (100 μg), whereas no cortisol response was observed in patients 1 and 3 (Table 2). Additional investigations performed in patients 2 and 4 revealed that iv injection of GnRH alone (100 μg) induced a significant increase in PCL reaching +99 and +254%, respectively (Fig. 2A). In patient 2, plasma LH concentration increased from less than 0.1 units/liter to 31 units/liter during the combined TRH/GnRH test, whereas it rose from 1.4 to 18 units/liter after GnRH injection. In the two patients, hCG (5000 IU im) also stimulated cortisol secretion (+342% in patient 2, and +101% in patient 4; Fig. 2A), whereas neither recombinant FSH (150 IU sc) nor TRH (250 μg iv) had any influence on PCL (data not shown). Incubation of cultured adrenocortical H2 and H4 cells with hCG (10^{-10} to 10^{-6} M) provoked a dose-dependent stimulation of corticosteroidogenesis (Fig. 2B). The pEC_{50} and efficacy (E_{max}) of hCG to stimulate cortisol production were 9.3 ± 0.4 and +243 ± 41%, respectively, in H2 cells and 9.3 ± 0.1 and +40 ± 2% in H4 cells (n = 4). In case 2, the maximum effect of hCG was obtained at a concentration of 10^{-8} M, and attenuation of the response was observed at higher concentrations of hCG (10^{-7} and 10^{-6} M). For concentrations up to 10^{-6} M, hCG had no effect on cortisol secretion from normal adrenocortical cells (Fig. 2B). Similarly, GnRH did not affect the secretory activity of H2 and H4 cells (Fig. 2B). The kinetics of the responses of perifused H2 and H4 explants to a pulse of hCG (10^{-6} M; 20 min) is shown in Fig. 2C. In both cases, hCG induced a rapid increase in cortisol secretion (+1300% for H2 cells and +507% for H4 cells), followed by a plateau phase with spontaneous oscillations of the secretion rate.

TABLE 2. Plasma cortisol responses to the clinical tests in the four patients with AIMAH

Patient	Meal	TRH/GnRH	Upright	Terlipressin	$5\text{-}HT_4$-R agonists	ACTH-(1–24)
1	−22	+6	+62	+52	+94[a]	+220
2	+486	+463	+29	−10	−70[a]	+33
3	−27	+5	+33	CI	+24[b]	+136
4	+381	+194	−15	+15	−7[b]	+415

The values correspond to the maximum plasma cortisol variations observed in the four patients in response to the screening tests, as described in *Patients and Methods*. The data are expressed as percentages of basal levels. Basal levels are calculated as the mean of two plasma cortisol values preceding each test. $5\text{-}HT_4$-R, $5\text{-}HT_4$ receptor. CI denotes that the test was contraindicated.

[a] Cisapride (10 mg orally).

[b] Metoclopramide (10 mg iv).

Effect of 5-HT on cortisol secretion

In patient 1, administration of cisapride (10 mg, orally) induced a significant increase in PCL (Table 2). A similar cortisol response was observed after a metoclopramide stimulation test (10 mg iv) performed during the follow-up of the patient (Fig. 3A). No stimulation of cortisol secretion was detected in patients 2 and 4 after administration of $5\text{-}HT_4$ receptor agonists (Table 2). Incubation of H1 cells with 5-HT (10^{-9} to 10^{-5} M) induced a dose-dependent stimulation of cortisol production (Fig. 3B). The potencies of 5-HT to stimulate corticosteroidogenesis in H1 cells and normal adrenocortical cells were similar (7.0 ± 0.1 and 7.3 ± 0.4, respectively; n = 4), but the efficacy of 5-HT to stimulate cortisol production was higher in H1 cells (+173 ± 6%) than in normal adrenocortical cells (+93 ± 12%; $P < 0.0001$).

Effect of posture test and posture-responsive hormones on cortisol secretion

A significant increase in PCL was observed during upright stimulation test in patients 1, 2, and 3 (Table 2 and Fig. 4A). Administration of the vasopressin analog terlipressin (0.5 mg iv) also stimulated cortisol secretion in patient 1 but not in patients 2 and 4 (Table 2 and Fig. 4A, *inset*). Incubation of Ang II (10^{-7} M) with H1, H2, H4, and normal adrenocortical cells induced a significant increase in cortisol secretion (Fig. 4B). The amplitude of the stimulation was significantly higher in cells derived from the hyperplastic tissues (H1 cells, +150 ± 41%; H2 cells, +202 ± 33%; H4 cells, +116 ± 7%) than in normal adrenocortical cells (+59 ± 9%). The β-adrenoreceptor isoproterenol (10^{-5} M) did not significantly modify cortisol secretion either from cultured H1, H2, and H3 cells or from normal adrenocortical cells (Fig. 4C). In contrast, incubation of H4 cells with isoproterenol provoked a +72 ± 3% increase in cortisol production. AVP (10^{-8} M) induced a significant increase in cortisol secretion from all types of cells (Fig. 4D). The amplitudes of the responses of H2 cells (+166 ± 28%), H3 cells (+75 ± 13%), and H4 cells (+51 ± 5%) were in the same range as those of normal cells (+87 ± 35%), whereas the amplitude of the response to AVP was significantly higher in H1 cells (+380 ± 85%). AVP was equally potent to stimulate cortisol production in H1 cells

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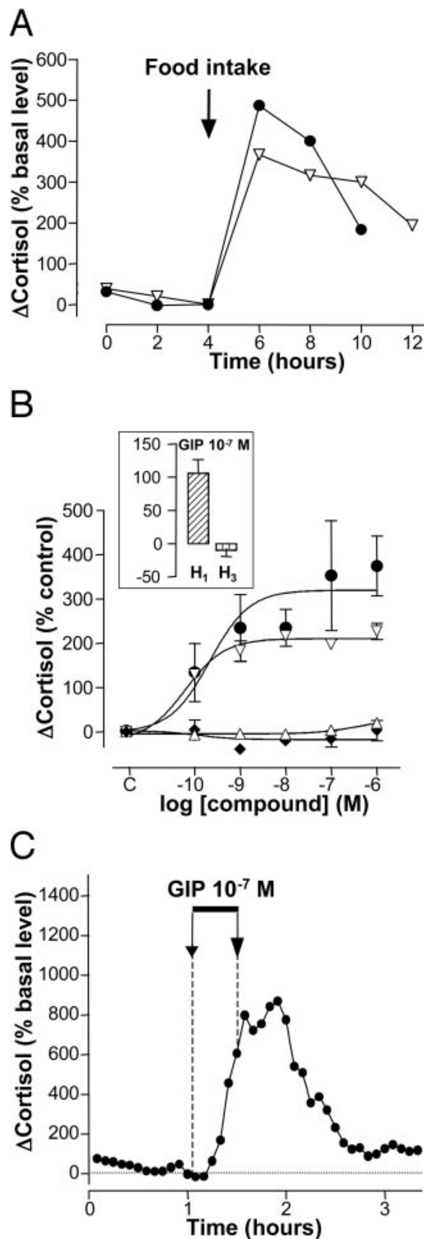

FIG. 1. Effects of food intake and gastrointestinal hormones on cortisol secretion from adrenal hyperplasias H2 and H4. A, Effect of food intake on PCLs in patients 2 (●) and 4 (▽). B, Effect of graded concentrations of GIP and GLP-1 (from 10^{-10} to 10^{-6} M) on cortisol production from cultured cells derived from hyperplasias H2 (●, GIP; and ◆, GLP-1) and H4 (▽, GIP; and △, GLP-1). *Inset*, Effect of GIP (10^{-7} M) on cortisol production from cultured cells derived from hyperplasias H1 and H3. C, Effect of a single pulse of GIP (10^{-7} M, 20 min) on cortisol production from perifused hyperplasia H2 explants. The spontaneous level of cortisol release (100% basal level) was calculated as the mean of the eight consecutive fractions preceding the pulse of GIP.

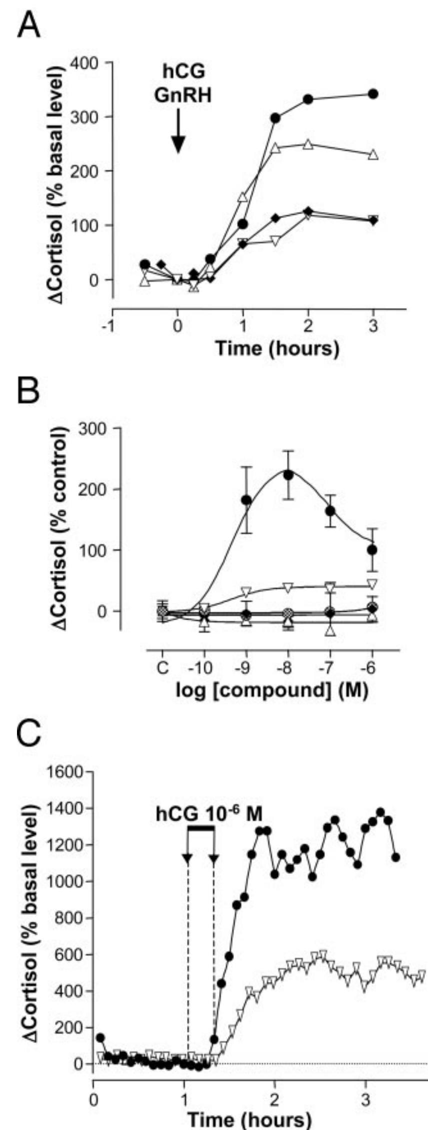

FIG. 2. *In vivo* and *in vitro* effects of GnRH and hCG on cortisol secretion from adrenal hyperplasias H2 and H4. A, Effects of GnRH (100 μg iv) and hCG (5000 IU im) on PCL in patients 2 (◆, GnRH; and ●, hCG) and 4 (△, GnRH; and ▽, hCG). B, Effects of graded concentrations of GnRH and hCG (from 10^{-10} to 10^{-6} M) on cortisol production from cultured cells derived from hyperplasias H2 (◆, GnRH; and ●, hCG) and H4 (△, GnRH; and ▽, hCG) and normal adrenocortical cells (◍, hCG). C, Effect of a single pulse of hCG (10^{-6} M, 20 min) on cortisol production from perifused adrenocortical explants removed from hyperplasias H2 (●) and H4 (▽). See Fig. 1 legend for other designations.

(pEC_{50} = 10.1 ± 0.1) and in normal cells (pEC_{50} = 9.7 ± 0.2) (Fig. 4D, *inset*).

Immunohistochemical detection of 5-HT and AVP

Incubation of H1 tissue slices with 5-HT antibodies produced intense labeling of the subcapsular region of the cortex (Fig. 5A). Clusters of immunoreactive cells were also observed in the central zones of hyperplastic nodules (Fig. 5B). 5-HT-positive cells had the morphological characteristics of spongiocytic cells, *i.e.* cells with abundant cytoplasm and numerous lipid droplets. Preincubation of the 5-HT antiserum with 5-HT (10^{-6} M) totally abolished immunostaining (Fig. 5C). Similarly, labeling of H1 tissue slices with AVP antibodies revealed the presence of immunoreactive material in the spongiocytic cells of the subcapsular region of the cortex (Fig. 5D). Small groups of immunoreactive cells were also detected in the hyperplastic nodules (Fig. 5E). In con-

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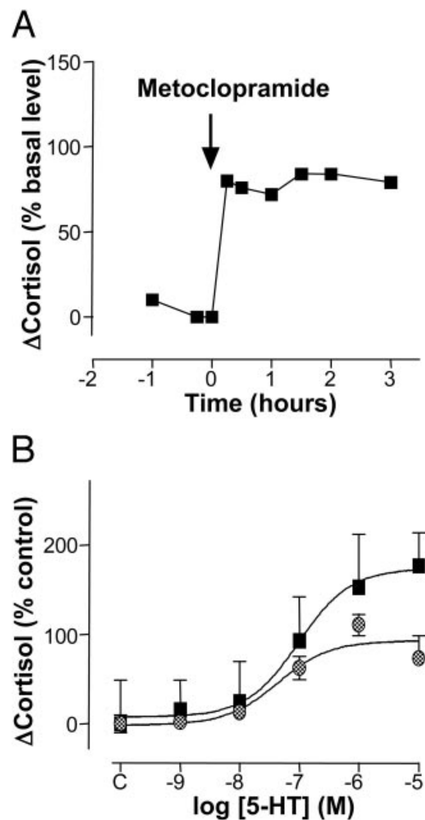

FIG. 3. *In vivo* and *in vitro* effects of $5\text{-}HT_4$ receptor agonists on cortisol secretion from adrenal hyperplasia H1. A, Effect of the $5\text{-}HT_4$ receptor agonist metoclopramide (10 mg iv) on PCL in patient 1. B, Effect of graded concentrations of 5-HT (from 10^{-9} to 10^{-5} M) on cortisol production from cultured cells derived from hyperplasia H1 (■) and normal adrenocortical cells (◍). See Fig. 1 legend for other designations.

trast, no labeling was observed when the AVP antibodies were preincubated with AVP (Fig. 5F).

Discussion

In AIMAHs causing subclinical and/or overt Cushing's syndrome, cortisol production can be controlled by multiple illegitimate membrane receptors for circulating and/or locally produced bioactive signals (1, 3, 4). In particular, a cortisol response to food intake associated with ectopic expression of the GIP receptor is relatively frequent in this condition (19). However, it is conceivable that food-induced stimulation of cortisol secretion could result from activation of other illegitimate receptors for gastrointestinal hormones, such as GLP-1. In our series of AIMAHs, cortisol secretion was stimulated by a mixed meal in two patients. In addition, *in vitro* studies showed that, in the two cases, food-induced cortisol response was mediated by GIP but not GLP-1, which failed to influence corticosteroidogenesis at concentrations up to 10^{-6} M. The fact that a cortisol response to GIP was observed in H1 cells, although patient 1 did not respond to food intake, shows that the lack of influence of meals on cortisol secretion does not eliminate an abnormal sensitivity of hyperplastic adrenal tissues to GIP. It also suggests that ectopic GIP receptors were expressed at a low level in the

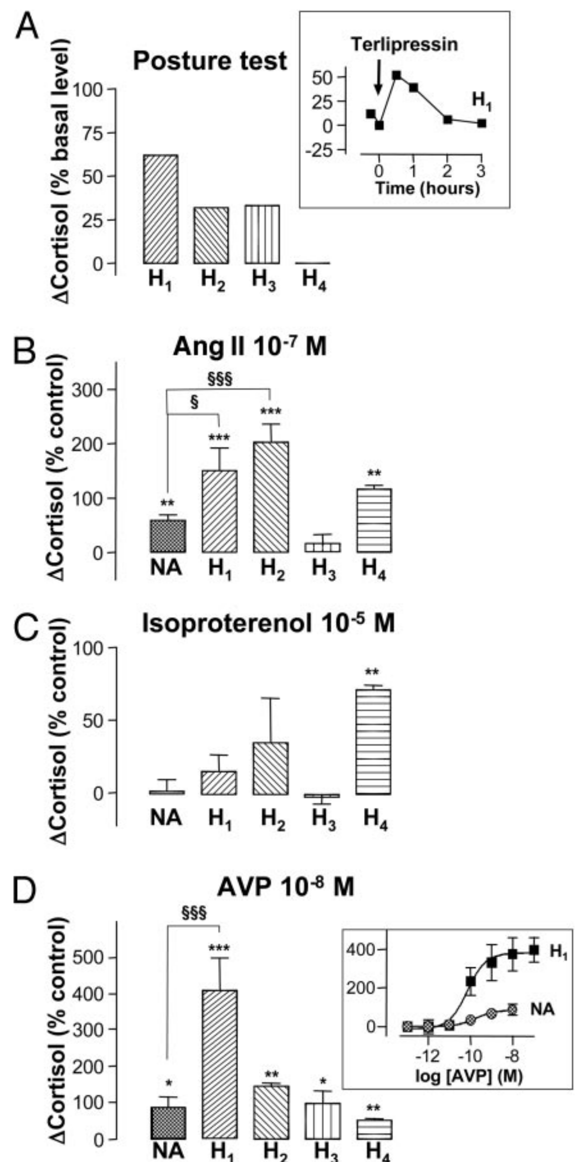

FIG. 4. Effects of posture test and posture-sensitive hormones on cortisol secretion for the four adrenal hyperplasias (H1–H4). A, Effect of a change from supine to upright posture on PCL in the four patients. *Inset*, Effect of terlipressin (10 mg, im) on PCL in patient 1. B–D, Effect of Ang II (10^{-7} M; B), isoproterenol (10^{-5} M; C), and AVP (10^{-8} M; D) on cortisol production from cultured cells derived from hyperplasias H1–H4 and normal adrenocortical (NA) cells. D, *Inset*, Effect of graded concentrations of AVP (from 10^{-12} to 10^{-7} M) on cortisol production from cultured cells derived from hyperplasia H1 (■) and NA cells (◍). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* control. §, $P < 0.05$; §§§, $P < 0.001$ *vs.* NA cells. See Fig. 1 legend for other designations.

adrenal tissue and thus could only be detected by a stimulation with high concentrations of GIP. In agreement with this hypothesis, postprandial plasma GIP levels usually reach a maximum of 0.2 nmol/liter (24), a concentration 500 times lower than that used in our *in vitro* study (10^{-7} M). Therefore, it will be interesting to measure the expression level of mRNA encoding the GIP receptor in H1 tissue. Perifusion studies revealed for the first time the kinetics of the cortisol response of hyperplastic adrenocortical tissue to a

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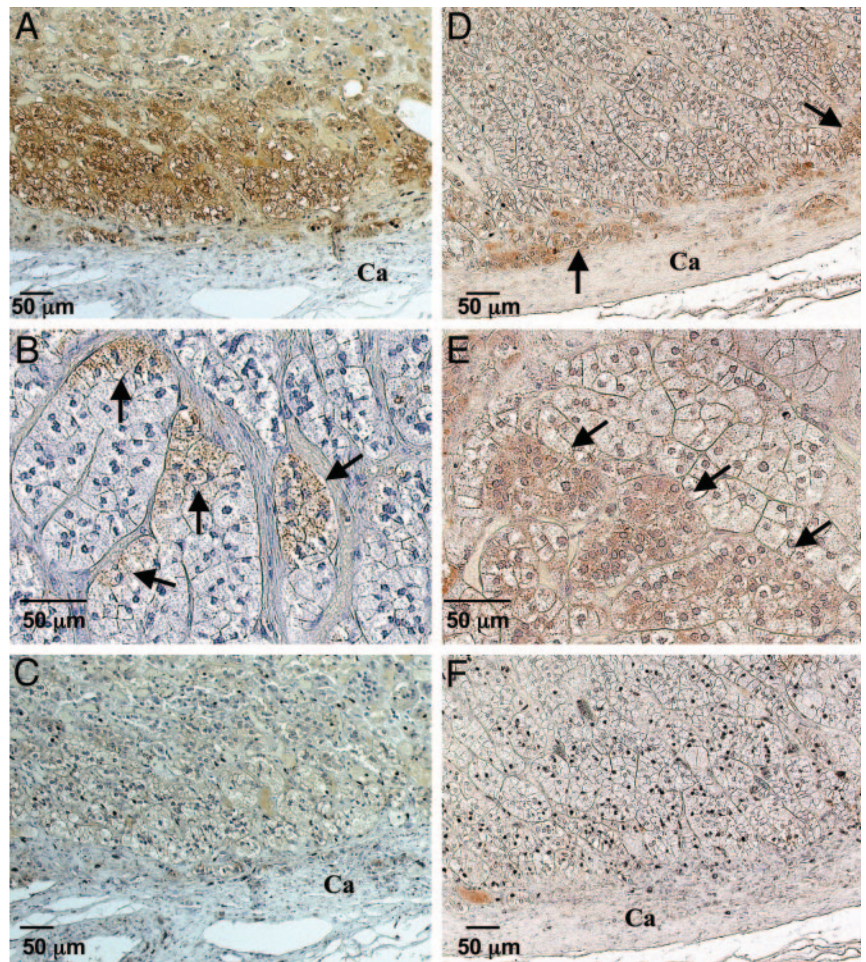

FIG. 5. Immunohistochemical localization of 5-HT and AVP in adrenal hyperplasia 1. A, 5-HT-positive cells in the subcapsular region of the hyperplastic cortex. B, Small group of 5-HT-positive cells (*arrows*) in the central region of a nodule of hyperplasia. C, Incubation of a consecutive section of the tissue with 5-HT antibodies preabsorbed with 5-HT (10^{-6} M) resulted in complete loss of the immunoreaction. D, AVP-positive cells in the subcapsular region of the hyperplastic cortex. E, Group of AVP-positive cells in a nodule of hyperplasia (*arrows*). F, Incubation of a consecutive section of the tissue with AVP antibodies preabsorbed with AVP (10^{-6} M) resulted in complete loss of the immunoreaction. Ca, Capsule. *Scale bars*, 50 µm.

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single pulse of GIP. The stimulatory effect of GIP was transient, indicating that the influence of food intake on PCL is probably limited to the meal and immediate postprandial periods; plasma GIP concentrations being very low in fasting conditions (~20 pmol/liter; Ref. 24). Finally, the relatively high fasting PCL observed in patient 4 suggests that other illegitimate receptors than the GIP receptor may play a significant role in the maintenance of cortisol secretion during interprandial periods.

The two hyperplasias sensitive to GIP also responded *in vivo* to combined administration of TRH and GnRH, GnRH alone, and hCG, but not to recombinant human FSH. The higher relative increase in PCL observed after concomitant injection of TRH and GnRH in patient 2, in comparison with the cortisol response induced by GnRH alone, likely resulted from the more pronounced GnRH-induced LH increase. Incubation of adrenocortical cells with hCG induced a dose-dependent increase in cortisol production, whereas GnRH had no effect on steroidogenesis in both cases. Thus, this study provides the first observation of AIMAHs responding to both LH/hCG and GIP. Conversely, the two tissues were unresponsive to 5-HT_4 receptor agonists, showing that an abnormal cortisol response to LH/hCG is not always associated with an increased sensitivity of AIMAH to serotonergic drugs, as suggested in a recent report (25). Consistent with a previous study showing that, in the human adrenal cortex, the LH/hCG receptor is almost exclusively expressed by zona reticularis (26), we did not observe any effect of hCG on cortisol production by normal adrenocortical cells. In one hyperplasia, the attenuation of the cortisol response observed with high concentrations of hCG suggests the occurrence of a desensitization phenomenon of the receptor, as previously shown in Leydig cells (27, 28). The molecular mechanisms involved in the prolonged stimulation of cortisol production evoked by a single pulse of hCG in perifused hyperplasia fragments remain unclear. In addition to its direct effect on steroidogenic cells, hCG may activate intraadrenal regulatory systems, which may in turn stimulate cortisol secretion.

It has previously been shown that the 5-HT_4 receptor, which is physiologically expressed in the human adrenal cortex, mediates the stimulatory effects of 5-HT on aldosterone and cortisol production (16, 29–31). It has also been observed that 5-HT_4 receptor mRNAs are overexpressed in most cisapride/metoclopramide-responsive AIMAHs causing subclinical and/or overt Cushing's syndrome (12). In agreement with these data, we found that the abnormal cortisol response to cisapride observed in one patient was associated with an increased efficacy of 5-HT to stimulate cortisol production *in vitro* from hyperplasia cells, in comparison with normal adrenocortical cells. The presence of 5-HT-like immunoreactivity, in a subpopulation of spongio-

cytic cells within the hyperplastic cortex, suggests that cortisol production may be under the control of an intraadrenal serotonergic tone involving an autocrine/paracrine mode of regulation. The occurrence of 5-HT in corticosteroidogenic cells has been previously reported in the rat adrenal gland, where glomerulosa cells are able to synthesize significant amounts of 5-HT through uptake and decarboxylation of circulating 5-hydroxytryptophan (32). However, in the normal human adrenal gland, 5-HT is exclusively stored in perivascular mast cells (16). Therefore, the present study provides the first demonstration of the ectopic localization of a paracrine regulatory factor, *i.e.* 5-HT, in a pathological adrenal tissue.

Three AIMAHs were shown to respond *in vivo* to the posture test. The increase in cortisol levels induced by a change from supine to upright posture may involve several hormones, including Ang II, catecholamines, and AVP (1). Thus, we have investigated *in vitro* the effect of these signals on cortisol production in cultured cells derived from the four tissues and from normal adrenocortical cells. As previously shown (17, 33, 34), the secretory activity of normal cells was stimulated by Ang II and AVP but was not influenced by the β-adrenoreceptor agonist isoproterenol. The data obtained *in vitro* from hyperplasia tissues were not strictly correlated with those of clinical testing. For instance, the *in vitro* sensitivities of the posture-responsive hyperplasia H3 to Ang II, isoproterenol, and AVP were similar to those of the normal adrenocortical tissue, whereas hyperplasia H4, which did not respond *in vivo* to the posture test, exhibited exaggerated *in vitro* responses to Ang II and isoproterenol. However, in hyperplasias 1 and 2, the results of cell incubation experiments clearly showed that the *in vivo* cortisol response observed during the upright stimulation test was mediated by Ang II, as previously suggested by Nakamura *et al.* (35), after *in vivo* pharmacological studies in a patient with posture-sensitive AIMAH. Similarly, the increased efficacy of AVP to stimulate cortisol production in H1 cells, in comparison with normal cells, which likely reflects an overexpression of vasopressin V_{1a} receptor mRNAs by the hyperplastic tissue (11), explains the abnormal *in vivo* cortisol response of patient 1 to terlipressin. Considering the low physiological plasma concentrations of AVP (10^{-12} M; Ref. 36), it is unlikely that circulating AVP may influence cortisol production from hyperplasia cells. Therefore, if AVP were to play a significant role in the pathophysiology of cortisol hypersecretion in AIMAH, the nonapeptide would have to be produced locally. The immunohistochemical results obtained from hyperplasia 1 confirmed the presence of AVP-like immunoreactivity in the tissue, consistent with a previous study showing the occurrence of proAVP mRNA in AIMAHs (11). Contrary to the normal adrenal gland, where AVP is stored in chromaffin cells (17), the hyperplastic cortex exhibited AVP-like immunoreactivity in a subpopulation of steroidogenic cells having the same tissular distribution as that of serotonergic cells. The detection of 5-HT and AVP in AIMAH spongiocytic cells provides additional evidence for the presence of hybrid cells, *i.e.* exhibiting both steroidogenic and neuroendocrine cell characteristics, in adrenocortical cortisol-producing lesions (23). It also indicates that AVP, like 5-HT, can act as an ectopic autocrine/paracrine regulatory factor in some AIMAH tissues. The role of this paracrine mode of communication in the maintenance of active corticosteroidogenesis will be explored *in vitro* and *in vivo*, by means of perifusion and clinical studies with specific vasopressinergic and/or serotonergic antagonists.

In conclusion, the present study additionally demonstrates that hyperplastic ACTH-independent adrenocortical tissues may simultaneously express multiple illegitimate membrane receptors for both circulating and intraadrenal paracrine/autocrine factors. In particular, we describe for the first time adrenocortical hyperplasias responding to both gonadotropin and GIP. Our data also demonstrate a hyperresponsiveness of AIMAH cells to 5-HT, Ang II, and AVP, in comparison with normal adrenocortical cells. Finally, they show for the first time that adrenal hyperplasias produce corticotropic substances on their own, providing evidence for autocrine/paracrine regulations of cortisol secretion within the tissues.

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