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Luteinizing Hormone (LH)-Responsive Cushing's Syndrome: The Demonstration of LH Receptor Messenger Ribonucleic Acid in Hyperplastic Adrenal Cells, which Respond to Chorionic Gonadotropin and Serotonin Agonists *in Vitro*

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In a substantial part of adrenal adenomas and hyperplasias from patients with Cushing's syndrome, cortisol production is controlled by the expression of aberrant hormone receptors on adrenocortical cells. We present *in vivo* and *in vitro* data of two patients with a LH-responsive Cushing's syndrome based on ACTH-independent bilateral adrenal hyperplasia.

Patients 1 and 2 are women who presented with Cushing's syndrome and bilateral adrenal hyperplasia. Endocrine testing demonstrated absence of cortisol diurnal rhythm, insufficient cortisol suppression after 1 mg dexamethasone orally, and undetectable ACTH levels in both patients. Both patients were treated by laparoscopic biadrenalectomy.

In *in vivo* testing, in patients 1 and 2, a profound cortisol rise was found after administration of GnRH [change in cortisol (ΔF), 118 and 106%, respectively], human CG (ΔF, 133 and 44%), LH (ΔF, 73 and 43%), ACTH (ΔF, 89 and 181%), and the 5-hydroxy-tryptamine receptor type 4 (5-HT_4) agonists cisapride (ΔF, 141 and 148%) and metoclopramide (ΔF, 189 and 95%).

In *in vitro* testing, adrenal cells from patient 2 responded, in a dose-dependent fashion, with cortisol production after exposure to human CG (ΔF, 45%), cisapride (ΔF, 68%), and metoclopramide (ΔF, 81%). ACTH induced cortisol production by cells from both patients (ΔF, 135 and 159%).

In receptor studies, LH receptor mRNA was demonstrated in adrenal tissue of both patients but also in control adrenal tissue of two patients with persisting pituitary-dependent Cushing's syndrome treated by biadrenalectomy. In neither patient were mutations found in the ACTH receptor gene.

LH-responsive Cushing's syndrome associated with bilateral adrenal hyperplasia may result from aberrant (or possibly increased) adrenal LH receptor expression. This variant is further characterized by adrenal responsiveness to 5-HT_4 receptor agonists, possibly pointing to an interaction between LH and serotonin in the regulation of cortisol secretion. Despite the regulatory potential of LH and 5-HT_4 receptor agonists on cortisol production in our patients, their adrenals seemed to be still sensitive to ACTH, both *in vivo* and *in vitro*. (*J Clin Endocrinol Metab* 88: 230–237, 2003)

ENDOGENOUS CUSHING'S SYNDROME is traditionally divided into ACTH-dependent and ACTH-independent variants. The spectrum of ACTH-independent Cushing's syndrome includes unilateral adrenal adenoma and carcinoma and, rarely, bilateral macro- or micronodular adrenal hyperplasia. Excess production of cortisol by adrenal tumors or hyperplasias was previously considered to be autonomous. However, in recent years, increasing evidence has been obtained that cortisol secretion by a substantial part of adrenal neo- or hyperplasias may be controlled by aberrant hormone receptors (1). This involves the adrenal expression of receptors (*e.g.* for catecholamines, TSH, vasopressin, and gastric inhibitory peptide), which appear to be functionally coupled to steroidogenesis.

Recently, Lacroix *et al.* (2) reported a female patient with bilateral adrenal hyperplasia and ACTH-independent Cushing's syndrome, which presented clinically only during pregnancy and after the menopause. In this patient, cortisol production *in vivo* was stimulated by LH, chorionic gonadotropin [human CG (hCG)], but also by agonists of the 5-hydroxy-tryptamine type 4 (5-HT_4) receptor, suggesting aberrant adrenal expression of LH and 5-HT_4 receptors. However, this was not confirmed by *in vitro* studies.

We describe two female patients with bilateral adrenal hyperplasia and ACTH-independent hypercortisolism who also exhibited a profound cortisol response to LH, hCG, and 5-HT_4 receptor agonists *in vivo*. Both patients were treated by laparoscopic biadrenalectomy, allowing for receptor and *in vitro* studies of hormone release. LH receptor mRNA was demonstrated in adrenal tissue of both patients. This indicates that in LH-responsive Cushing's syndrome associated with bilateral adrenal hyperplasia, cortisol production is controlled via adrenal LH receptor expression.

Abbreviations: APC, Adenomatous polyposis coli; ΔF, change in cortisol; hCG, human CG; 5-HT_4, 5-hydroxy-tryptamine receptor type 4; PRL, prolactin; RNase, ribonuclease.

Subjects and Methods

Case reports

Patient 1. The first patient is a 60-yr-old postmenopausal woman who was admitted for analysis of bilateral adrenal hyperplasia. During an urological evaluation for transient hematuria, abdominal computed tomography revealed bilaterally enlarged adrenals (left adrenal, 5 cm; right adrenal, 3.5 cm). Clinical symptoms suggestive of hypercortisolism included easy bruisability, muscle weakness, and depression. The medical history included a hysterectomy. No abnormal weight gain had occurred during two previous pregnancies. She had not been treated with glucocorticoids. She used estrogen-replacement therapy. There was no family history of endocrine disorders. At physical examination, she had moderate central obesity, mild plethora, and skin atrophy with several ecchymoses. The patient's height was 1.67 m, and her weight was 68 kg. Blood pressure was 130/85 mm Hg. Endocrine evaluation (see *Results*) revealed ACTH-independent hypercortisolism, with stimulation of cortisol production after the administration of GnRH, hCG, LH, cisapride, metoclopramide, and ACTH, but no response to FSH. A trial with leuprolide acetate was proposed to the patient, but she insisted on having an operation. Subsequently, the patient was treated by laparoscopic biadrenalectomy. Histological examination of adrenal tissue showed micro- and macronodular hyperplasia of the adrenal cortex, with a predominance of macronodules. The size of the nodules varied from 2 mm to 2 cm, and there was no internodular atrophy. Postoperatively, replacement therapy was started with hydrocortisone, 9α-fluorhydrocortisone, and dehydroepiandrosterone. Clinical symptomatology of Cushing's syndrome fully resolved after several months.

Patient 2. The second patient is a 40-yr-old premenopausal woman who presented with a 12-month history of easy bruisability, proximal muscle weakness, and hypertension. In the past 10 yr, she had recurrent episodes of depression and weight gain of approximately 40 kg. In the second and fourth year of this period, the patient had two pregnancies without an abnormal weight gain. She had undergone a hysterectomy, 2 yr before admission. There was no family history of endocrine abnormalities. The patient's height was 1.59 m, and her weight was 83 kg. Blood pressure was 140/90 mm Hg. She had mild facial plethora, a cushingoid fat distribution with a buffalo hump, supraclavicular fat pads, and central obesity but no acne, hirsutism, or abdominal striae. Endocrine testing (see *Results*) demonstrated ACTH-independent hypercortisolemia. An abdominal computed tomography scan showed bilateral adrenal hyperplasia (left adrenal, 3 cm; right adrenal, 5 cm). Additional tests demonstrated a marked cortisol response after administration of GnRH, hCG, LH, cisapride, and metoclopramide as well as ACTH, but no response to FSH. The patient refused a trial with leuprolide acetate and was therefore treated by laparoscopic biadrenalectomy, followed by replacement therapy with hydrocortisone, 9α-fluorhydrocortisone, and dehydroepiandrosterone. Histological examination of the adrenal glands showed micro- and macronodular hyperplasia of the adrenal cortex, with the size of the nodules varying from 2 mm to 3 cm. There was no internodular atrophy. Her clinical picture of Cushing's syndrome disappeared within several months thereafter. Apart from this diagnosis, this patient was diagnosed with familial adenomatous polyposis, associated with a mutation in the adenomatous polyposis coli (APC) gene, with localization of adenocarcinomas in the duodenum, cecum, and rectum. The association of familial adenomatous polyposis, adrenal neoplasia, and APC mutation has been described previously (3). After the biadrenalectomy, she underwent a Whipple procedure and a total colectomy.

Control patients. Hyperplastic adrenal tissue, serving as control tissue, was obtained from two female patients of 45 and 56 yr old, respectively, with persistent Cushing's disease after pituitary surgery and radiotherapy, who were treated by laparoscopic biadrenalectomy.

Procedure

All studies were performed according to the rules of the hospital medical ethics committee. Informed consent was obtained from all patients.

Assays. Plasma cortisol (reference value, 200–800 nM), urinary cortisol (determined in unextracted urine; upper limit, 850 nmol/24 h), and plasma ACTH-(1–39) concentrations (detection limit, 10 ng/liter) were measured by a fluorescent immunoassay (Diagnostic Products, Los Angeles, CA). Plasma LH (premenopausal reference value, 1–50 IU/liter; postmenopausal, 15–90 IU/liter) and FSH (premenopausal, 1–25 IU/liter; postmenopausal, 35–150 IU/liter) concentrations were determined by an immunofluorometric assay (Diagnostic Products). Plasma estradiol concentrations (premenopausal, 50–800 pM; postmenopausal, <100 pM) were measured by a RIA (Diagnostic Products).

Cell preparation and incubation studies. Directly after laparoscopic removal, adrenal cortical tissue of the major part of the adrenals was minced into small pieces and dissociated with collagenase (type I; Sigma, St. Louis, MO) as described previously (4, 5). Cell viability was determined by trypan blue staining and was more than 80%. Subsequently, the cells were resuspended in incubation medium (DMEM with 0.2% BSA) and incubated with and without test substances during 2 or 24 h. These incubations were performed in quadruplicate using 500,000 cells/ml. After 2- or 24-h incubation, 0.5 ml distilled water was added to each tube, and the resulting suspension was stored at –20 C until measurement of hormone concentrations, as described previously (4). The 2-h incubations were performed with the following substances: ACTH-(1–24) (Synacthen; Novartis, Basel, Switzerland; concentrations: 32, 64, 132, and 615 pg/ml), forskolin in a concentration of 1 μM, recombinant human GnRH (gonadorelin; Aventis Pharma, Frankfurt, Germany; 0.1 and 1 μM), recombinant human LH (Serono, Rome, Italy; 10, 50, 100, and 1,000 mIU/ml), purified hCG (Organon, Oss, The Netherlands; 10, 50, 100, and 1,000 mIU/ml), recombinant human FSH (Serono, Italy; 10, 50, and 100 mIU/ml), cisapride (Janssen Pharmaceuticals, Cilag, The Netherlands; 0.01, 0.1, and 1 μM), metoclopramide (0.1, 1, and 10 μM), and the combinations of ACTH (64 pg/ml) with: GnRH (1 μM), LH (100 mIU/ml), hCG (100 mIU/ml), cisapride (0.1 μM), and metoclopramide (1 μM). The 24-h incubations were performed with ACTH (615 pg/ml), forskolin (1 μM), LH (100 mIU/ml), hCG (100 mIU/ml), metoclopramide (1 μM), and (in patient 2) also with estradiol (Steroloids, Wilton, NH; 0.01 and 0.1 μM), activin A (25 ng/ml), and inhibin A (25 ng/ml).

Detection of LH receptor mRNA. After laparoscopic biadrenalectomy, adrenal specimens were frozen immediately in liquid nitrogen and stored at −80 C. Total RNA was isolated from adrenal specimens using an LiCl-urea protocol (6). For detection of LH receptor mRNA expression, using a ribonuclease (RNase) protection assay, a 300-bp RNA probe was used, transcribed from a 300-bp (bp, 1100–1400) blunted Bsu36I-XbaI fragment that was cloned in the SmaI-XbaI sites of the pBluescript vector. This probe identifies part of exon 11 of the LH receptor gene. Other probes corresponding to exons 1–10 of the LH receptor were also employed but were less efficient (lower signal; results not shown). As control for RNA isolation, expression of γ-actin mRNA was used as described before (7). As positive control for the RNase protection assay, RNA isolated from a stable cell line expressing the human LH receptor was used. HEK293 cells were transfected with the expression vector pSG5, containing human LH receptor cDNA (8) in combination with the cAMP-response reporter pCRE6Lux (8, 9). Several stable lines were obtained with various levels of LH receptor expression and response to hCG. Clone K15 was selected, because this clone showed low LH receptor mRNA expression, as compared with the other clones combined with a solid response to hCG (Dr. A. van Marle, unpublished results). After RNase expression, the bands were quantified using a Phosphoimager and ImageQuant software package (Molecular Dynamics, Inc./B&L Systems, Zoetermeer, The Netherlands). mRNA expression is depicted as the ratio of the human LH receptor over γ-actin.

Characterization of the ACTH receptor gene sequence. Leukocyte DNA was prepared from both patients by standard procedures. Genomic DNA was amplified and sequenced using a Big dye protocol (Applied Biosystems, Warrington, UK) analyzed on an ABI 377 automated DNA sequencer. PCR primers were described previously (10).

Statistics. The effects of various test substances on *in vitro* cortisol production by cultured adrenal cells were tested by ANOVA, followed by Newman-Keuls test.

Results

Hypercortisolism

Patient 1. The initial endocrine evaluation revealed an absent cortisol diurnal rhythm: at 0800 h, 339 nM; at 1700 h, 352 nM; at 2200 h, 363 nM; and at midnight, 441 nM. The patient's cortisoluria varied from 550–630 nmol/24 h. The cortisol concentration at 0800 h, after oral administration of 1 mg dexamethasone at 2200 h, was 336 nM (suppression cutoff value, <150 nM) and at 7 h after 7 mg dexamethasone iv (11), 370 nM (no decrease). Basal ACTH concentrations were below the detection limit of 10 ng/liter.

Patient 2. Endocrine evaluation demonstrated absence of cortisol diurnal rhythm: at 0800 h, 365 nM; at 1700 h, 402 nM; and at 2200 h, 404 nM. Cortisoluria was increased and varied between 900-1800 nmol/24 h. Plasma cortisol concentrations were not suppressed after the oral administration of 1 mg dexamethasone (cortisol at 0800 h, 469 nM), or by iv administration of 7 mg dexamethasone (cortisol after 7 h, 630 nM). Basal ACTH concentrations were undetectable. Administration of 1 μg/kg human CRH iv was not, followed by a response of plasma ACTH and cortisol concentrations.

Effects of GnRH, hCG, LH, and FSH

Data are shown in Table 1 and Figs. 1 and 2.

Patient 1. Administration of 100 μg GnRH iv resulted in a rapid increase in LH levels from 11.4 U/liter to peak levels after 60 min, directly followed by an increase in cortisol concentrations from 444 nM to peak levels after 150 min (Fig. 1A). FSH levels rose more slowly from 26.4 to 58.3 U/liter after 150 min. Plasma estradiol concentrations, 633 pM at baseline, did not change significantly after GnRH administration. Administration of 10,000 U hCG im induced a comparable increase in plasma cortisol levels from 448 nM to peak levels after 240 min (Fig. 1B). After administration of 300 U LH im cortisol, concentrations rose from 181 nM to peak levels after 30 min (Fig. 1C).

Patient 2. After administration of 100 μg GnRH iv, cortisol levels increased from 379 nM to peak levels after 90 min, preceded by a rise in LH concentrations from 2.8 U/liter to peak levels after 60 min (Fig. 2A). FSH concentrations increased from 5.1 to 29.9 U/liter after 120 min. No change was observed in plasma estradiol concentrations, 344 pM at baseline, after GnRH administration. Administration of 10,000 U hCG im resulted in an increase in plasma cortisol levels from 445 nM to peak levels after 240 min (Fig. 2B). After administration of 300 U LH im, cortisol concentrations rose from 332 nM to peak levels after 90 min (Fig. 2C). FSH administration (300 U im) was not followed by a cortisol response in either patient (data not shown). In both patients, ACTH levels remained undetectable in all stimulation tests.

TABLE 1. Peak plasma cortisol values, as percentage of baseline values, in *in vivo* stimulation tests in two patients with LH-responsive Cushing's syndrome based on bilateral adrenal hyperplasia

Stimulation test	Patient 1 (peak value as % of baseline)	Patient 2 (peak value as % of baseline)
GnRH (100 μg iv)	218	206
hCG (10,000 U im)	233	144
LH (300 U im)	173	143
FSH (300 U im)	95	99
Cisapride (10 mg orally)	241	248
Metoclopramide (10 mg orally)	289	195
ACTH (250 μg im)	189	281

Effects of ACTH, cisapride, and metoclopramide

Patient 1. After the administration of 10 mg cisapride orally, plasma cortisol levels increased from 572 nM to peak levels after 180 min (Fig. 1E). Three hours after the administration of cisapride, an ACTH stimulation test (250 μg im) was performed. Cortisol levels subsequently rose further, from 1400 to 3369 nM, after 120 min (Fig. 1D). The oral administration of 10 mg metoclopramide was followed by an increase in cortisol levels from 395 to 1140 nM (Fig. 1F).

Patient 2. Stimulation with 250 μg synthetic ACTH im resulted in a cortisol rise from 290 to 1234 nM after 90 min (Fig. 2D). Administration of cisapride and metoclopramide was followed by increases in plasma cortisol levels from 357 to 885 nM (Fig. 2E) and from 278 to 543 nM (Fig. 2F), respectively, after 120 min. Plasma ACTH levels were undetectable in all stimulation tests in both patients.

Effects of other stimuli

Both patients were screened for the presence of other abnormal cortisol responses based on aberrant adrenal receptors, according to the protocol of Lacroix *et al.* (1), including serial measurements of ACTH and cortisol in the fed and fasting state and a posture test (2 h, supine position), followed by a 2-h ambulatory period, a standard mixed meal, a 200-μg TRH test, a 1-mg glucagon test, and a 10-IU arginine vasopressin test. No abnormal cortisol responses were found in these tests (data not shown).

In vitro studies

Two-hour incubation. Data are shown in Tables 2 and 3. Cultured adrenocortical adenoma cells, obtained from patients 1 and 2 and control patients 1 and 2, produced 36 ± 1.7, 91.5 ± 1.1, 122.8 ± 10.6, and 85.2 ± 5.9 nmol cortisol/tube (mean ± SD), respectively, after 2 h. Incubation with ACTH stimulated cortisol production in a dose-dependent fashion, to maximum values of 84.4 ± 12.6, 236.8 ± 4.1, 468.6 ± 54.8, and 232.7 ± 26.5 nmol/tube, respectively. Forskolin, as stimulator of cAMP production, stimulated *in vitro* cortisol production by the adrenal cells of all patients (Table 2). Cisapride and metoclopramide had a dose-dependent effect on cortisol production by cells obtained from patient 2 but no effect on cortisol production by cells from patient 1 or the control patients (Table 2). GnRH, LH, hCG, and FSH had no significant effect on cortisol production in patient 1 and the control patients, whereas hCG stimulated cortisol production in patient 2 (Table 3). ACTH-stimulated cortisol production was not potentiated by coincubation with GnRH, LH, hCG, cisapride, or metoclopramide (data not shown).

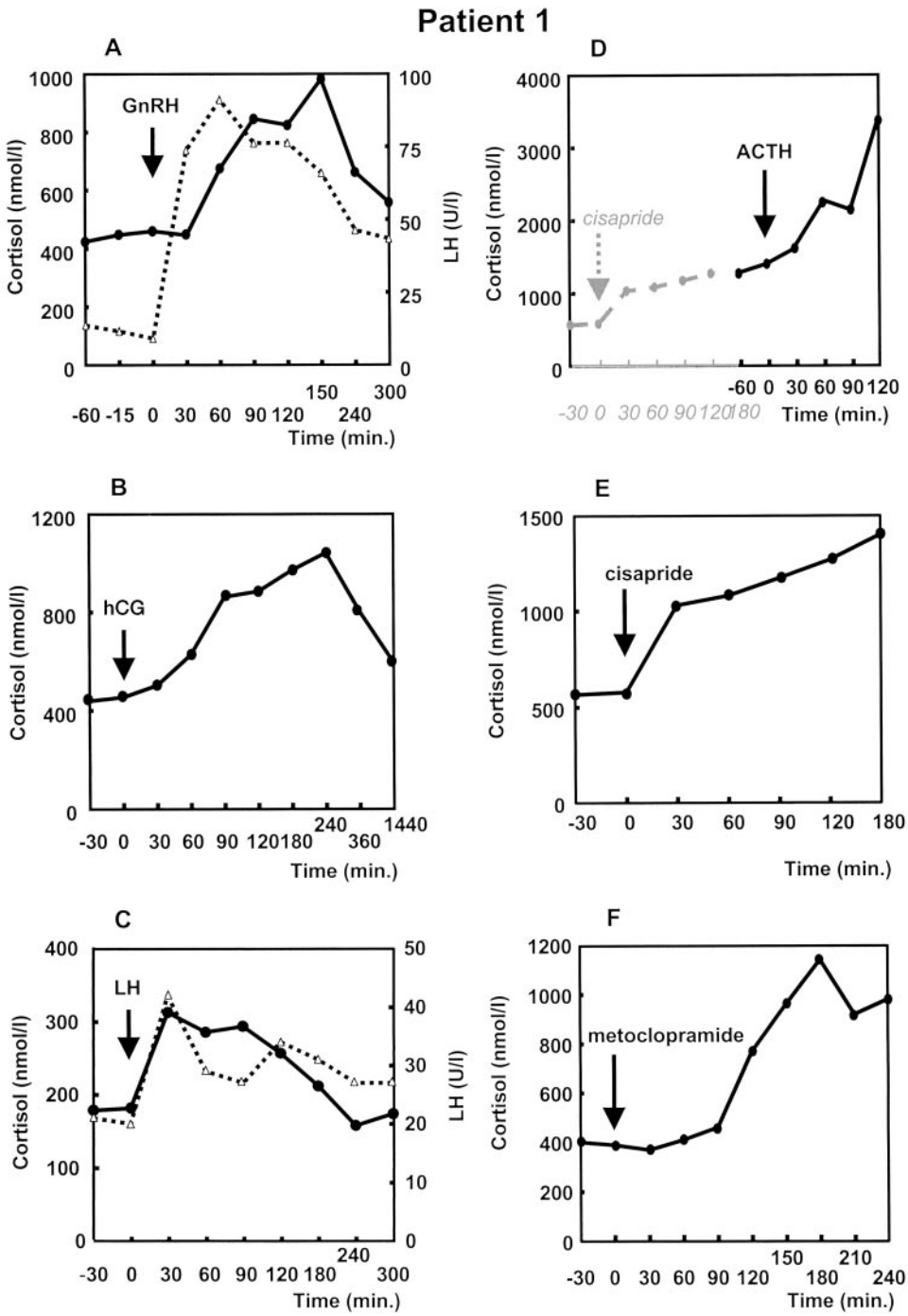

FIG. 1. *In vivo* plasma cortisol responses, in patient 1, to 100 μg GnRH iv (A), 10,000 U hCG im (B), 300 U LH im (C), 250 μg ACTH im (D), 10 mg cisapride orally (E), and 10 mg metoclopramide orally (F). A and C, *Solid lines* represent plasma cortisol concentrations, and *dashed lines* represent plasma LH concentrations.

Twenty-four-hour incubation. Incubation of cultured adrenal cells during 24 h with the same panel of test substances had no additive effect on cortisol production (data not shown). The adrenal cell culture of patient 2 was also incubated with estradiol, activin, and inhibin during 24 h, which had no significant effect on *in vitro* cortisol production.

Receptor studies

LH receptor. Presence of LH receptor mRNA was demonstrated in the hyperplastic adrenal tissue of patients 1 and 2, as well as in hyperplastic adrenal tissue of both control patients with pituitary-dependent Cushing's syndrome (Fig. 3). LH receptor mRNA expression in adrenal tissue from both the patients with LH-responsive Cushing's syndrome and the control patients was low, compared with the LH-receptor-expressing K15 cell line that served as control. LH receptor mRNA expression was slightly higher in adrenal tissue from the patients with LH-responsive Cushing's syndrome, compared with adrenal tissue from the control patients.

ACTH receptor. The ACTH receptor coding region, as determined by PCR of genomic DNA obtained from peripheral

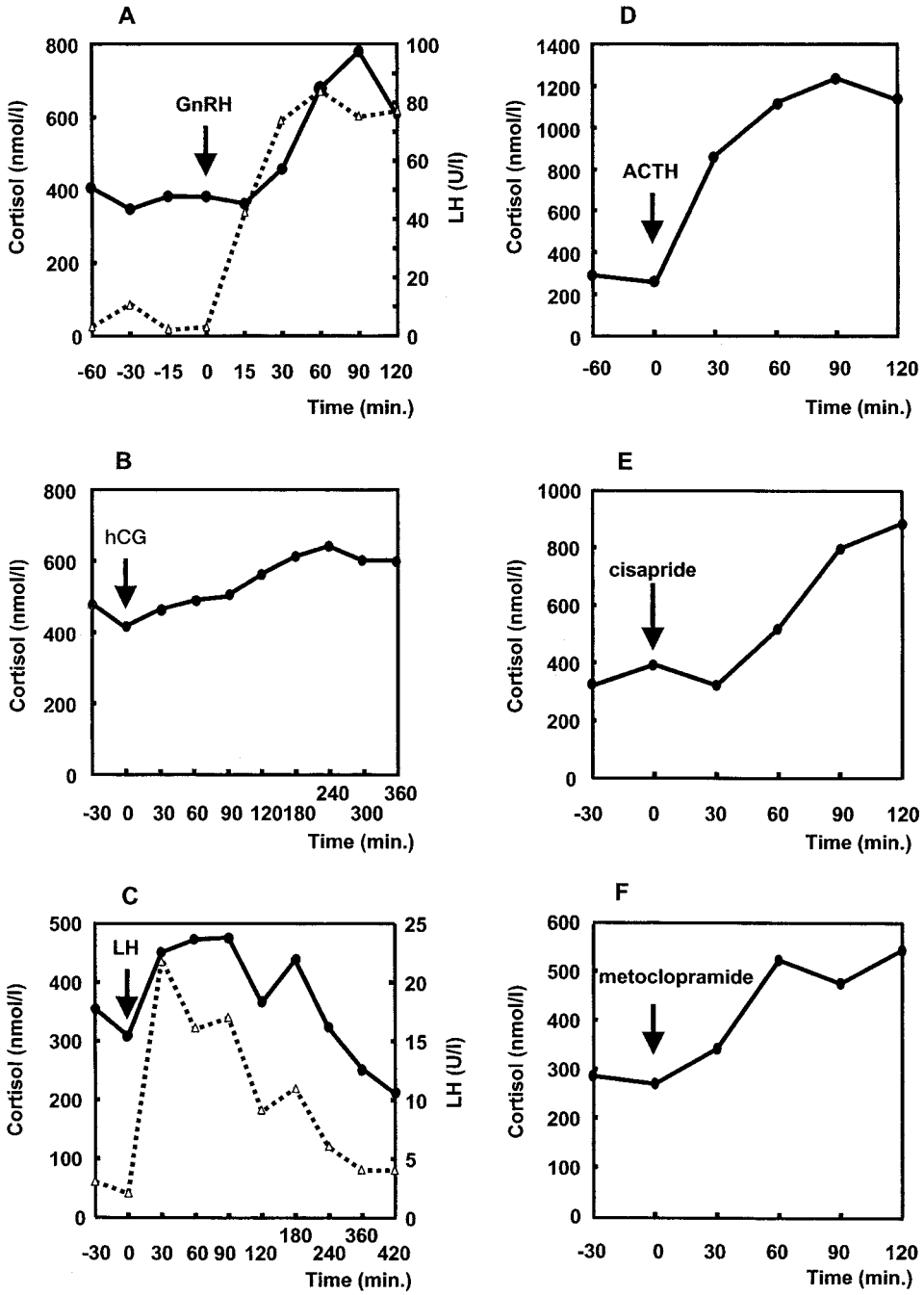

FIG. 2. *In vivo* plasma cortisol responses, in patient 2, to 100 μg GnRH iv (A), 10,000 U hCG im (B), 300 U LH im (C), 250 μg ACTH im (D), 10 mg cisapride orally (E), and 10 mg metoclopramide orally (F). A and C, *Solid lines* represent plasma cortisol concentrations, and *dashed lines* represent plasma LH concentrations.

leukocytes, was entirely normal in both patients (data not shown).

Discussion

The ACTH-independent Cushing's syndrome in both our patients was based on bilateral adrenal hyperplasia and characterized by *in vivo* responsiveness of cortisol production to GnRH, hCG, LH, and 5-HT_4 receptor agonists. The cortisol response to GnRH, hCG, and LH, but not to FSH, as well as the presence of LH receptor mRNA in adrenal tissue suggest that LH regulates, at least in part, cortisol production in these patients directly at the adrenocortical level.

To date, one case has been previously reported of a postmenopausal woman with a LH/5-HT_4 receptor agonist-responsive Cushing's syndrome resulting from bilateral adrenal hyperplasia (2). This patient was treated with the GnRH analog leuprolide acetate, resulting in suppression of LH levels, followed by normalization of cortisol production. This implies that the cortisol response to LH indeed plays a pathogenetic role in this form of Cushing's syndrome, rather than being an epiphenomenon. This previous patient and our patient 1 were postmenopausal, which might point to a certain threshold for plasma LH concentrations to induce hypercortisolism. However, patient 2 was premenopausal,

TABLE 2. *In vitro* cortisol responses of cultured adrenal adenoma cells to ACTH, forskolin, cisapride, and metoclopramide (2-h stimulated cortisol values, as percentage of patient's own control values)

Test substance	Patient 1	Patient 2	Control 1	Control 2
Control	100 ± 2	100 ± 3	100 ± 3	100 ± 4
ACTH				
32 pg/ml	134 ± 7[a]	152 ± 4[a]	370 ± 15[a]	162 ± 2[a]
64 pg/ml	215 ± 7[a]	190 ± 9[a]	382 ± 17[a]	204 ± 8[a]
132 pg/ml	212 ± 10[a]	216 ± 5[a]	376 ± 14[a]	247 ± 10[a]
615 pg/ml	235 ± 17[a]	259 ± 4[a]	369 ± 21[a]	291 ± 16[a]
Forskolin 1 μM	308 ± 20[a]	355 ± 7[a]	223 ± 10[a]	204 ± 8[a]
Cisapride				
0.01 μM	84 ± 5	90 ± 5	89 ± 1	93 ± 3
0.1 μM	92 ± 6	157 ± 7[a]	101 ± 6	98 ± 4
1 μM	96 ± 5	168 ± 11[a]	109 ± 7	103 ± 7
Metoclopramide				
0.1 μM	103 ± 4	105 ± 5	77 ± 2	91 ± 3
1 μM	95 ± 2	150 ± 5[a]	114 ± 3	105 ± 2
10 μM	98 ± 6	181 ± 8[a]	92 ± 5	90 ± 2

Adrenocortical adenoma cells were prepared from hyperplastic adrenal glands of two patients with LH-responsive Cushing's syndrome and of two control patients with pituitary-dependent Cushing's syndrome. Data are expressed as mean (n = 4) ± SEM.

[a] $P < 0.01$, compared with patient's own control.

TABLE 3. *In vitro* cortisol responses of cultured adrenal adenoma cells to GnRH, LH, hCG, and FSH (2-h stimulated cortisol values, as percentage of patient's own control values)

Test substance	Patient 1	Patient 2	Control 1	Control 2
Control	100 ± 2	100 ± 3	100 ± 3	100 ± 4
GnRH				
0.1 μM	93 ± 4	93 ± 5	90 ± 2	107 ± 4
1 μM	93 ± 6	93 ± 7	97 ± 4	109 ± 2
LH				
10 mIU/ml	80 ± 2	101 ± 6		88 ± 5
50 mIU/ml	89 ± 5	101 ± 1		90 ± 4
100 mIU/ml	94 ± 4	81 ± 4	92 ± 4	92 ± 9
1000 mIU/ml	92 ± 5	110 ± 5	101 ± 5	103 ± 4
hCG				
10 mIU/ml	92 ± 9	93 ± 3		105 ± 6
50 mIU/ml	91 ± 3	90 ± 3		98 ± 2
100 mIU/ml	96 ± 6	122 ± 6[a]	100 ± 9	101 ± 4
1000 mIU/ml	98 ± 5	145 ± 8[a]	102 ± 6	103 ± 5
FSH				
10 mIU/ml	102 ± 5	108 ± 4		109 ± 2
50 mIU/ml	98 ± 4	103 ± 6	98 ± 4	104 ± 4
100 mIU/ml	105 ± 6	99 ± 6	101 ± 5	111 ± 5

Adrenocortical adenoma cells were prepared from hyperplastic adrenal glands of two patients with LH-responsive Cushing's syndrome and of two control patients with pituitary-dependent Cushing's syndrome. Data are expressed as mean (n = 4) ± SEM.

[a] $P < 0.05$, compared with patient's own control.

which indicates that LH concentrations in the lower range may also regulate cortisol production in the presence of adrenal LH receptor expression.

The LH receptor is, in man, predominantly expressed in gonadal tissues but has also been found in other organs, such as the uterus, brain, and hypothalamus (12). We demonstrated LH receptor mRNA in the adrenal tissue of both patients, suggesting aberrant LH receptor expression. However, in the adrenal tissues of our two patients with pituitary-dependent Cushing's syndrome, we found LH receptor mRNA as well. This is in accordance with a recent postmortem study showing that normal human adrenal tissue contains both LH receptor mRNA and the LH receptor protein, located in the zona fasciculata and zona reticularis (13). These data indicate that the LH receptor may be constitutively expressed by the adrenal gland. LH-responsive Cushing's syndrome may therefore be caused by an up-regulation of the otherwise weakly expressed LH receptor. Postulated mechanisms underlying an increased expression of the LH receptor gene include gene rearrangement, resulting in fusion of the LH receptor gene with an adrenocortical-specific promotor, and abnormalities in transcription factors or their coactivators or corepressors, caused by gain- or loss-of-function mutations (1). Alternatively, a mutation of the LH receptor may underlie adrenal responsiveness to LH. Future studies should include measurement of the LH receptor protein and assessment of the LH receptor DNA sequence in adrenal tissue from patients with LH-responsive Cushing's syndrome and in control adrenal tissue.

There is increasing evidence that LH has regulatory effects on adrenal steroidogenesis. For instance, hCG, acting via the LH receptor, stimulates dehydroepiandrosterone sulfate production by human fetal adrenal cells (14) and induces cortisol secretion by guinea pig adrenal cells (15). In addition, in women with the polycystic ovarian syndrome, elevated LH concentrations coincide with an increased adrenal androgen production (16). Kero *et al.* (17) examined the effects of LH on adrenal function, using a transgenic mouse model with a high constitutive LH production. These bLHβ-CTP mice develop polycystic ovaries with increased androgen and estrogen levels. Interestingly, chronically elevated LH concentrations in these mice are also accompanied by the development of bilateral adrenal hyperplasia with a concomitantly increased (up to 14-fold) glucocorticoid production. *In vitro* studies showed the presence of LH receptor mRNA and protein in adrenal tissue of bLHβ-CTP mice. Accordingly, hCG induced, in a dose-dependent fashion, corticosterone production by primary adrenal cells prepared from these mice. However, elevated LH concentrations are not the only factor regulating adrenal corticosterone production in bLHβ-CTP mice. First, gonadectomy of nontransgenic mice, also leading to elevated LH levels, did not result in increased corticosterone production. Second, if the female bLHβ-CTP mice were gonadectomized, serum corticosterone levels decreased to levels found in nontransgenic mice, and no adrenal response to hCG was found *in vitro*. This

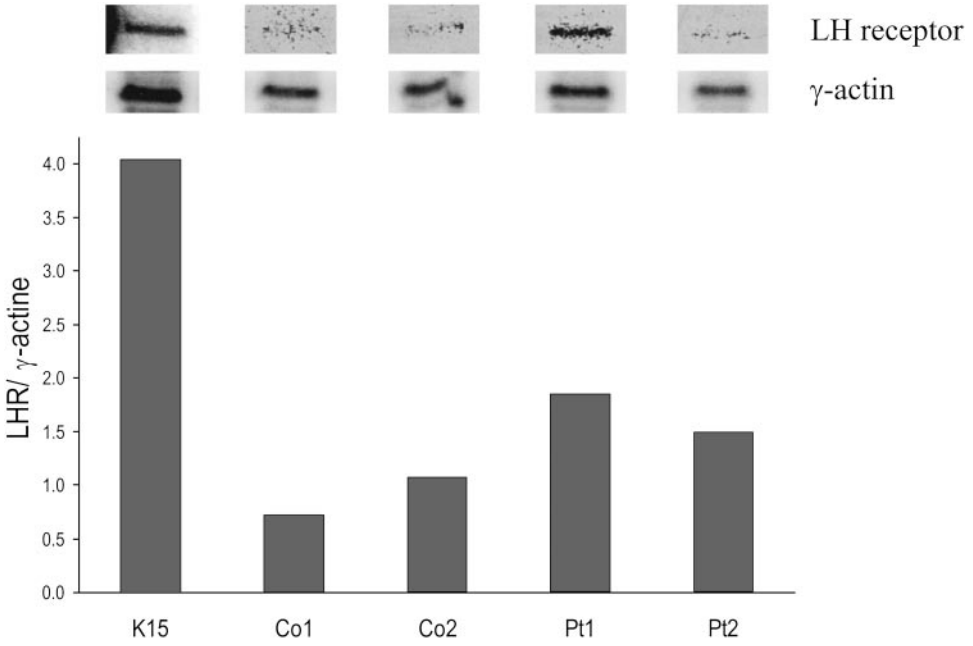

FIG. 3. Human LH receptor mRNA expression in adrenal tissue. Using RNase protection assay, LH receptor mRNA expression was determined in adrenal tissue obtained from two control patients (Co1 and Co2) with pituitary-dependent Cushing's syndrome and from two patients with LH-responsive Cushing syndrome (Pt1 and Pt2) based on bilateral adrenal hyperplasia. As control, the LH receptor-expressing K15 cell line was used. In the *top* of the figures, the autoradiograph of the specific bands are shown. The *bars* indicate the quantitative analysis of the bands, expressed as the ratio of LH receptor to γ-actin.

implies that a gonadal factor derived from the dysfunctional ovaries is involved in LH-mediated corticosterone production in bLHβ-CTP mice. In this respect, it was postulated that increased estrogen levels induce PRL secretion (17). PRL, in turn, is known to induce LH receptor expression in rodents. In our two patients, serum PRL levels were normal.

Another experimental animal model involves neutered ferrets. Hyperadrenocorticism, based on adrenal adenoma or hyperplasia, is a common finding in gonadectomized ferrets (18). GnRH stimulation in these ferrets results in an increased adrenal steroid hormone production, and LH receptors have been demonstrated immunohistochemically in adrenal tissue of neutered ferrets (N. J. Schoemaker, personal communication). Thus, the gonadectomy-induced increase in LH release may lead to adrenal LH receptor expression and LH-responsive steroidogenesis. Indeed, hyperadrenocorticoid ferrets can successfully be treated with leuprolide acetate (19). Interestingly, both our patients and the patient described by Lacroix *et al.* (2) had undergone a hysterectomy. However, only the patient reported by Lacroix *et al.* had also undergone a bilateral oophorectomy; she was treated though with estrogen-replacement therapy.

Although we found a profound cortisol response to LH (agonists) *in vivo* in our patients, *in vitro*, only high dosages of hCG induced a significant increase in cortisol production by adrenal cells obtained from patient 2. Considering the low LH receptor mRNA content, this may be explained by a low LH receptor density. Further, it cannot be excluded that only a fraction of the adrenals obtained from patient 1, perhaps one or several nodules, was LH responsive, although the major part of the adrenals was homogenized before the cells were brought into culture. Alternatively, LH may interact with additional factors in the regulation of adrenal cortisol production, not present in the *in vitro* setting of dispersed cells in which part of the *in vivo* existing interactions are lost. First, based on the studies with bLHβ-CTP mice, an ovarian factor may be involved that may influence LH receptor expression. Incubation with estradiol, activin, or inhibin, however, did not influence cortisol response. Future studies should include coincubation of adrenal cells with LH and ovarian hormones.

Second, the effect of LH on adrenal steroidogenesis may be potentiated by or mediated via serotonin. It is intriguing that the LH-responsive Cushing's syndrome in both the previously described patient (2) and in our two patients is associated with an *in vivo* cortisol response to 5-HT_4 receptor agonists. In addition, Lacroix and co-workers (20) described two patients with subclinical Cushing's syndrome and bilateral adrenal hyperplasia who also showed an *in vivo* cortisol response to hCG and 5-HT_4 receptor agonists. In the adrenal gland, serotonin is produced by mast cells that can regulate steroid hormone production in a paracrine manner (21, 22). Serotonin stimulates aldosterone secretion via activation of the 5-HT_4 receptor, which is expressed in the zona glomerulosa but also, to a lesser extent, in the zona fasciculata (21). In healthy subjects, 5-HT_4 receptor agonists induce a rise in aldosterone but not in cortisol levels (21). Locally produced serotonin may be a permissive factor for LH-induced cortisol secretion. It might also be speculated that LH induces intraadrenal serotonin release. On the other hand, 5-HT_4 receptor agonists, by themselves, were also capable of inducing cortisol production, which suggests an altered functional coupling between the 5-HT_4 receptor and cortisol secretion and/or an increased 5-HT_4 receptor expression by cortisol-secreting cells. Serotonin may also influence cortisol production indirectly via induction of intraadrenal IL-6 release (21). Similar to the responses to LH, however, we found a discrepancy between the *in vivo* and *in vitro* responses to 5-HT_4 receptor agonists; only in patient 2, an effect of cisapride and metoclopramide was demonstrated *in vitro*. Therefore, an interaction between LH and serotonin and between their (post) receptors (effects) in the regulation of cortisol production seems likely. The exact mechanism, though, is presently unknown.

One of the proposed mechanisms of a predominant adrenal response to stimuli other than ACTH is a defect in the ACTH signal cascade, *e.g.* caused by a mutation of the ACTH receptor (1). The ACTH receptor coding region, as determined in genomic DNA, was, however, entirely normal in both patients. Indeed, despite the regulatory potential of LH and 5-HT_4 receptor agonists on cortisol production in our patients, their adrenals were still responsive to ACTH, both *in vivo* and *in vitro*.

In conclusion, LH-responsive Cushing's syndrome associated with bilateral adrenal hyperplasia may result from aberrant or increased adrenal LH receptor expression. This variant is further characterized by adrenal responsiveness to 5-HT_4 receptor agonists, possibly pointing to an interaction between LH and serotonin in the regulation of cortisol secretion.

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