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Estradiol Stimulates Cortisol Production by Adrenal Cells in Estrogen-Dependent Primary Adrenocortical Nodular Dysplasia

OMAR CATICHA, WILLIAM D. ODELL, DANA E. WILSON, LYNN A. DOWDELL, ROBERT H. NOTH, ARTHUR L. M. SWISLOCKI, JACQUES J. LAMOTHE, AND ROGER BARROW

Department of Internal Medicine, Endocrinology Division, University of Utah School of Medicine (O.C., W.D.O., D.E.W.), Salt Lake City, Utah 84132; Veterans Administration Medical Center (L.A.D., R.H.N., A.L.M.S., J.J.L.), Martinez, California 94553; the Departments of Internal Medicine (L.A.D., R.H.N., A.L.M.S., J.J.L.), Family Practice (R.B.), and Ambulatory Family Medicine (R.B.), University of California School of Medicine, Davis, California 95616; and Merrithew Memorial Hospital (R.B.), Martinez, California 94553

ABSTRACT

Adrenal glands from a patient with ACTH-independent Cushing's syndrome, whose symptoms worsened during pregnancy and oral contraceptive use, were cultured in different concentrations of estradiol. Estradiol stimulated cortisol secretion in a dose-response manner in the absence of ACTH. Since immunoglobulins G from this patient did not stimulate corticosterone production in a mouse adrenal bioassay, an adrenal-stimulating immunoglobulin is unlikely to be the cause of adrenal hyperfunction in this case. This is the first description of estradiol stimulation of cortisol production by cultured adrenal cells in ACTH-independent Cushing's syndrome. (*J Clin Endocrinol Metab* **77:** 494–497, 1993)

PRIMARY adrenocortical nodular dysplasia (PAND) is a rare maldevelopment resulting in autonomous hyperfunction of the adrenal cortex. Usually the disorder manifests itself in childhood or the peripubertal years (1). Symptoms and laboratory findings are typical of ACTH-independent Cushing's syndrome, and the treatment of choice is bilateral adrenalectomy (2).

De Moor *et al.* (3) previously described a patient with PAND who had disappearance of symptomatology after removal of one adrenal gland, followed by pituitary irradiation. This patient had a transient relapse coinciding with pregnancy.

We have studied a patient with PAND, whose symptoms and findings of Cushing's syndrome were documented during two pregnancies and estrogen-progestin treatment. She was treated during her last pregnancy with bilateral adrenalectomy. We report herein that estradiol directly stimulated cortisol production of these abnormal glands *in vitro*. The possibility of an autoimmune origin in this case is remote, since immunoglobulins G (IgGs) isolated from this patient did not stimulate steroidogenesis in an *in vitro* mouse adrenal cell bioassay.

Materials and Methods

Assays of patient's blood and urine

Assays on samples collected before the third pregnancy were performed either in the clinical laboratory of the V.A. Medical Center (Martinez, CA; serum cortisol) or in a contract laboratory (Smith Kline Bioscience Laboratories, Dublin, and Van Nuys, CA). Serum cortisol was measured by fluorescence polarization immunoassay (Abbot TDx, North Chicago, IL); urinary free cortisol was measured by RIA. Creatinine excretion was measured in all urine collections to confirm the adequacy of the sample; all values ranged between 0.9–1.1 g/24 h. Assays on samples collected during and after the third pregnancy were performed at the Salt Lake City V.A. Medical Center.

Adrenal research studies

All research methods applied to the adrenal glands and Ig bioassays were performed at the University of Utah.

Tissue culture

Portions of the adrenal cortex were immediately plated on RPMI (Flow Laboratories, McLean, VA) and used for the preparation of isolated cells. Minced tissue was incubated with 0.1% trypsin (Sigma Chemical Co., St. Louis, MO) and 0.01% DNAse (Sigma) until the cells were dispersed. The cells were then filtered through sterile nylon mesh to remove connective tissue and washed with RPMI. After centrifugation at 100 × *g*, cells were plated in 24-well polystyrene plates (5×10^5 cells/well) into RPMI with 2.5% fetal calf and 15% horse serum. The medium was changed every 4 days. Estradiol (Sigma) was dissolved in ethanol, filtered, and added to each well in concentrations ranging from 0–30 ng/mL. The final ethanol concentration was 0.01% (vol/vol) in all wells. Each concentration of estradiol was added in duplicate, and cells were incubated at 37 C in 95% O_2-5% CO_2 atmosphere for 4 days. The media were removed and frozen at −20 C until assayed for cortisol. These cells survived in culture 20 days, and all experiments were performed before completing the first 10 days of culture.

Mouse adrenal ACTH bioassay

An *in vitro* dispersed adrenal cell ACTH bioassay previously described from our laboratory (4) was used to study the potential capability of

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Address all correspondence and requests for reprints to: Omar Caticha, M.D., Department of Internal Medicine, University of Utah School of Medicine, 50 North Medical Drive, Salt Lake City, Utah 84132.

serum and purified IgGs from the patient to stimulate corticosterone production. For each assay, 10 male mice were killed by cervical dislocation between 0800–0900 h.

Corticosterone (for the mice bioassay) was measured by RIA using a rabbit anticorticosterone antiserum, B3–163 (Endocrine Sciences, Tarzana, CA; final concentration, 1:10,000), [1,2,6,7-^{3}H]corticosterone (New England Nuclear Research Products, Wilmington, DE; SA, 88 Ci/mmol), and corticosterone (Sigma). Bound and free were separated using 100 μL 1:5 diluted goat antirabbit serum, and after centrifugation, aliquots of the supernatant (free) were mixed with liquid scintillation cocktail (Beckman, CA) and counted in a β-counter. All assays were performed in duplicate. The sensitivity of the assay was 300 pg/mL, and the intraassay variation coefficient was 14.6%.

Cortisol was measured in duplicate using solid phase kits (Coat-a-Count, Diagnostic Products Corp., Los Angeles, CA) with a sensitivity of 2 ng/mL and an intraassay variation coefficient of 2.37%. Estradiol in the highest concentration present in the different media assayed (30 ng/mL) did not influence the cortisol standard curves (data not shown).

Preparation of IgG

The patient's serum was passed over a Cl-4B column containing protein-A-Sepharose (Pharmacia, Uppsala, Sweden), and the IgG was prepared according to a previously described method (6). Control IgG preparations were obtained from two normal subjects and tested in multiple doses in duplicate in the ACTH bioassay.

Statistical analysis

Cortisol production in wells exposed to different concentrations of estradiol and the effect of IgGs on corticosterone production in the mouse bioassay were tested by one-factor analysis of variance; $P < 0.05$ was regarded as significant.

Case Report

The clinical and laboratory data are summarized in Table 1.

A 33-yr-old caucasian female had menarche at age 13 yr, with regular menses until her first pregnancy at age 25 yr. Her family history was unremarkable. No member had hirsutism or Cushing's syndrome. Sporadically she received different treatments for hypertension. Her first pregnancy was complicated by preeclampsia, and during her second pregnancy, she was referred to the V.A. Medical Center (Martinez, CA) for evaluation of hypertension and purple striae. Cushing's syndrome was suspected and was confirmed by clinical and laboratory means. Computerized tomography of the hypothalamus/pituitary area and adrenals was normal; the adrenals were well visualized and were normal in size and configuration. Chest x-ray showed vertebral compression fractures, but revealed no mass lesions. During the second trimester, she underwent petrosal venous sampling at the University of California Medical Center in San Francisco; all ACTH values were at the lower limit of detectability. She delivered at 33 weeks gestation, and the infant had several malformations. After delivery, her symptoms rapidly regressed and 24-h urinary cortisol and serum cortisol levels returned to normal ranges. However, dexamethasone suppression tests showed slight paradoxical increase in cortisol levels.

After several months free of symptoms or signs, the patient requested contraception and was placed on Lo/Ovral 21 (0.3 mg norgestrel and 0.03 mg ethinyl estradiol). One month later, Cushing's syndrome returned. Repeat adrenal computerized tomography was normal, and laboratory data confirmed the clinical diagnosis. A [^{125}I]iodocholesterol scan showed bilateral adrenal hyperfunction. The oral contraceptives were discontinued, and approximately 40 days later, 24-h urinary cortisol measurements were again within the normal range, with disappearance of the Cushingoid features. There did not appear to be symptoms or findings of Cushing's syndrome during different phases of the menstrual cycle. Bilateral adrenalectomy was recommended, but the patient moved to Utah without acceding to these recommendations.

Eleven months later, she became pregnant, and at the first month of gestation, symptoms and signs of Cushing's syndrome again developed. Laboratory data were also compatible, and bilateral adrenalectomy was performed during the 18th week. The adrenal glands were enlarged, weighing 14.0 g (left) and 12.0 g (right). They were studded with multiple small nodules. The patient was treated with replacement hydrocortisone and 9α-fluorohydrocortisone, and has remained well, without Cushing's symptoms, to the present time. The adrenal glands and serum were used to perform the studies described.

Sections of both left and right adrenal glands showed marked cortical nodular hyperplasia (Fig. 1). The histological description of these glands was indistinguishable from those previously described (2).

Results

The cortisol secreted by the *in vitro* cultured cells from the patient exposed to different concentrations of estradiol is shown in Fig. 2. Cortisol secretion increased strikingly in a dose-dependent manner with increasing estradiol concentrations ($P < 0.0001$ for each gland). Both adrenals showed similar behavior.

TABLE 1. Summary of clinical and laboratory data

Clinical information	Clinical presentation	24-h urinary cortisol (nmol/day)	Cortisol, AM (nmol/L)	ACTH (pmol/L)	Dexamethasone test: cortisol (nmol/L)	
					1 mg	8 mg
Before age 25 yr	Sporadic hypertension					
1st pregnancy	Preeclampsia, premature labor					
1st postpartum	Asymptomatic					
2nd pregnancy	Cushing's syndrome	7118 (55–248)	1195 (193–662) 1142 (193–662)	6.3 (0–18)	1210	1173
2nd postpartum	Asymptomatic	77 (0–226)	198 (193–662) 189 (193–662)	5.5 (0–18)	237 204	
Lo/Ovral 21	Cushing's syndrome	1517 (0–226)	1076 (193–662) 1158 (193–662) 2455 (post-dex)			
Postcontraception	Asymptomatic	33 (0–226) 58 (0–226) 135 (0–226)				
3rd pregnancy	Cushing's syndrome	1620 (55–248)	1235 (220–689)	6.1 (0–18)	1250	
Postsurgery	Asymptomatic	62 (55–248)	245 (220–689)			

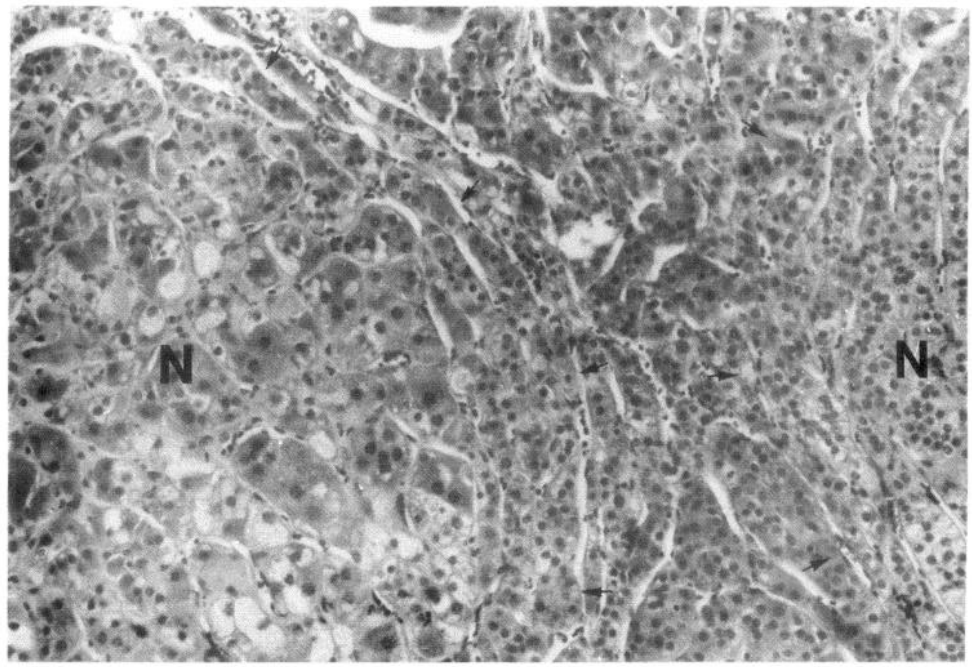

FIG. 1. Right (R) adrenal of the patient. This section shows marked cortical nodular hyperplasia in two adjacent nodules (N). *Arrows* show edges of the nodules. Stained with hematoxylin and eosin; original magnification, ×400.

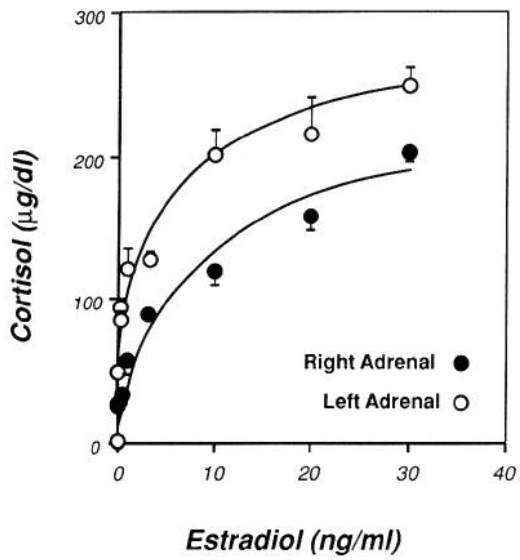

FIG. 2. Influence of estradiol on cortisol production *in vitro* by the patient's adrenal glands. *Bars* represent the SD of the value obtained in wells with the same estradiol concentration. The increase in cortisol production according to estradiol concentration is significant ($P < 0.0001$ for each gland).

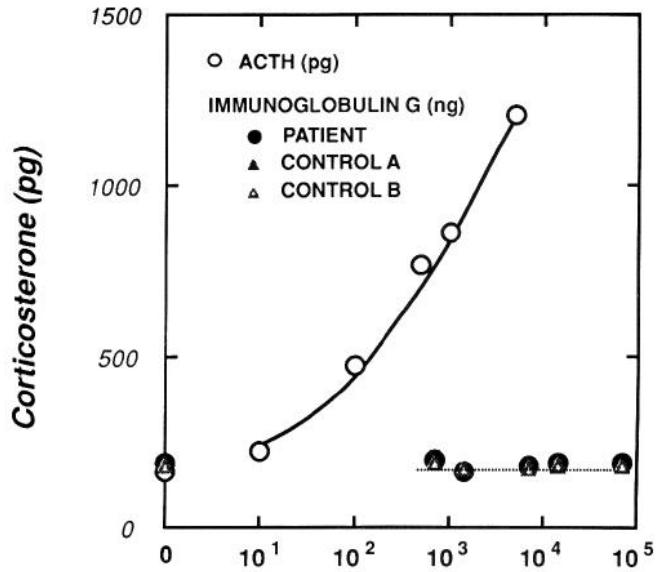

FIG. 3. Corticosterone production in the mouse adrenal bioassay. Mass (per tube) of IgG (nanograms) or ACTH (picograms) is shown on the *abscissa*. ACTH stimulated corticosterone in a dose-response manner ($P < 0.001$), whereas IgGs obtained from two normal subjects and the patient did not influence corticosterone production ($P > 0.25$).

Corticosterone stimulation by synthetic ACTH in mouse adrenal cells is shown in Fig. 3. Corticosterone production rose in a dose-dependent manner between 0–5000 pg/tube ACTH ($P < 0.001$). None of the IgGs tested in different concentrations stimulated corticosterone production ($P > 0.25$). Estradiol in the same concentrations as those used for the human PAND cells also failed to stimulate mouse adrenal cells.

Discussion

Analysis of previous reports of PAND suggest that it may not be a single disorder. It appears to be a congenital disorder, manifesting itself either in childhood or at puberty (2).

In 1965, De Moor *et al.* (3) reported a patient with PAND who developed Cushing's syndrome during pregnancy. Aside from the patient reported in the present study, no other patient has been described in whom Cushing's syndrome appeared both during pregnancy and oral contraceptive use. Since Van Berkhout *et al.* (5) and Wulffraat *et al.* (6) suggested that PAND might be an autoimmune disorder, analogous to Graves' disease, we studied this patient's IgG for the ability to stimulate adrenal steroidogenesis. No such effect was detectable, even when using IgG concentrations

as high as 100 μg/mL. The failure of the patient's IgG to stimulate corticosterone production from the *in vitro* cultured cells does not definitely preclude the possibility that the pathogenesis of PAND in this patient was originally autoimmune.

The patient's adrenal glands were strikingly affected by estrogen both *in vitro* and *in vivo*, and possibly also responded paradoxically to dexamethasone *in vivo*. The amount of estradiol produced by the patient's ovaries during her normal menstrual cycle was inadequate to stimulate glucocorticoid production to abnormal levels. However, higher estrogen concentrations, associated with oral contraceptives and normal pregnancy, stimulated cortisol production to pathological levels, producing Cushing's syndrome.

Other researchers (7, 8) reported adrenal tumors that paradoxically respond to dexamethasone suppression tests. In our case, the patient had increased cortisol levels after receiving standard doses of dexamethasone. In the patient described here, cortisol excretion increased paradoxically, but remained within normal ranges in response to dexamethasone when studied during remission after the second pregnancy. These observations suggest that the patient's adrenal glands were also stimulated by dexamethasone. Unfortunately, the cultures of adrenal glands from this patient did not survive, and additional studies designed to clarify the mechanism of estradiol stimulation of cortisol secretion or the effects of dexamethasone and other hormones could not be carried out. It is important to point out that estradiol, in the concentrations used in the present study, did not modify corticosterone production in the mouse adrenal bioassay (data not shown). It is also interesting that rats treated with estrogens *in vivo* had decreased corticosteroid production, but such effects were dependent upon the presence of the pituitary (9–11).

Since the cultured adrenal cells were studied in the first 10 days of culture, we ruled out the possibility of cortisol release by mechanisms other than active secretion, such as cell lysis and death. During the experiments, the cells were alive and attached to the plates.

We speculate that PAND is associated with a wide range of estrogen sensitivity among various patients. In patients with onset at puberty, the concentrations present during the normal menstrual cycle might produce Cushing's syndrome. Our patient would be less sensitive to estrogen than the previously described patients with pubertal onset of Cushing's syndrome. As additional patients are seen by other clinicians, we hope that further studies can be carried out to answer these questions.

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