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Expression of 11β-Hydroxysteroid Dehydrogenase Type 2 in an ACTH-producing Small Cell Lung Cancer

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Non-pituitary tumors that produce adrenocorticotropic hormone (ACTH) exhibit resistance to the normal feedback effects of glucocorticoids on proopiomelanocortin (POMC) gene expression. This glucocorticoid resistance is typically complete, although some tumors show only relative glucocorticoid resistance in the clinical setting. The molecular mechanisms responsible for these clinical pathophysiologic observations are unknown, but might include glucocorticoid receptor defects or aberrant expression of enzymes or transporters that exclude glucocorticoids from access to their intracellular receptors. We examined whether ACTH-producing non-pituitary tumor cells might express 11β-hydroxysteroid dehydrogenase (11β-HSD), the principal 'gatekeeper' enzyme known to metabolize glucocorticoids. 11β-HSD mRNA and enzyme activity were assessed in DMS-79 cells, a line derived from an ACTH-producing small cell lung cancer. RT–PCR studies showed expression of mRNA encoding 11β-HSD2 but not 11β-HSD1 in DMS-79 cells. Control human fibroblasts expressed predominantly 11β-HSD1 but also had detectable 11β-HSD2 mRNA, while HepG2 hepatoma cells also expressed only 11β-HSD2 mRNA. Whole cell assays in DMS-79 cells revealed 11β-HSD activity with a K_m for cortisol of 26.1 ± 9.0 nM and V_{max} of 57.0 ± 5.9 pmol/h/mg protein. HepG2 cells expressed a similar high affinity enzyme activity, while control fibroblasts expressed 11β-HSD activity with a K_m for cortisol of 652 nM. Conversion of cortisol to cortisone in DMS-79 cells was inhibited to 7% of baseline by addition of 10 μM glycyrrhetinic acid. Dexamethasone (20 nM) was converted to a single product in DMS-79 cells at a rate of 17.2 pmol/h/mg protein; this activity was also inhibited by glycyrrhetinic acid. We conclude that DMS-79 cells express 11β-HSD2. While DMS-79 cells harbor additional defects in glucocorticoid signaling, these data suggest that expression of 11β-HSD2 might contribute to the development of the glucocorticoid-resistant phenotype of some ACTH-producing tumors. © **1998 Published by Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Ectopic ACTH syndrome, due to production of bioactive products of the proopiomelanocortin gene (POMC) by non-pituitary tumors [1–3] is characterized by ACTH-dependent hypercortisolism that is resistant to normal physiologic suppression by glucocorticoids [4]. While this fact has been exploited clinically for more than thirty-five years as the basis for the high-dose dexamethasone suppression test, the molecular basis for this glucocorticoid resistance has only recently begun to be elucidated. In general, cell lines established from such non-pituitary neoplasms that secrete ACTH also display *in vitro* the glucocorticoid resistance evidenced *in vivo*. We have recently shown one such cell line, DMS-79, to express abnormal transcripts of the glucocorticoid receptor (GR) that encode a protein lacking much of the steroid-binding domain [5, 6]. However, in addition to such abnormalities in GR-mediated signaling, other mechanisms might also contribute to the development of the glucocorticoid-resistant phenotype. One possible mechanism could involve the enzyme 11β-hydroxy-

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Table 1. *Sequence of oligonucleotide primers used for reverse transcription and PCR*

	11β-HSD1
5′ sense	5′-CTC GAG TCG GAT GGC TTT TTA TG-3′
3′ antisense	5′-ACT TGC TTG CAG AAT AGG-3′
	11β-HSD2
5′ sense	5′-ACC GTA TTG GAG TTG AAC AGC-3′
3′ antisense	5′-TCA CTG ACT CTG TCT TGA AGC-3′
	Sodium–potassium ATPase
5′ sense	5′-ATA TGG AAC AGA CTT GAG CCG-3′
3′ antisense	5′-GGC AAT TCT TCC CAT CAC AGT-3′

Primers used were those designed by Whorwood *et al.* [12].

steroid dehydrogenase (11β-HSD) that mediates conversion of the bioactive 11-hydroxy glucocorticoids (e.g., cortisol in humans) to their inactive 11-keto form (e.g., cortisone).

Any candidate enzyme postulated to be involved in the generation of glucocorticoid resistance in ACTH-dependent Cushing's syndrome would need to meet several criteria suggested by available clinical data. Such an enzyme must have high affinity for cortisol, must be capable of metabolizing dexamethasone, and would expected to function predominantly as a dehydrogenase. While 11β-HSD1 lacks these characteristics, 11β-HSD2, cloned and characterized from several species, has been found to have these properties [7–9]. Normally expressed in the classic mineralocorticoid target tissues of the kidney and distal colon, 11β-HSD2 is NAD^+ dependent, exclusively dehydrogenase in directionality, and has a high affinity for cortisol (K_m of 35–47 nM). 11β-HSD2 is inhibited by glycyrrhetinic acid and carbenoxolone and is capable of metabolizing the synthetic glucocorticoid dexamethasone [7–9].

We postulated that expression of 11β-HSD2 by ACTH-producing neoplastic cells might contribute to the glucocorticoid-resistant phenotype displayed by such tumors. Here we present data showing that DMS-79 cells, derived from an ACTH-producing small cell lung cancer, express this enzyme.

MATERIALS AND METHODS

Cell culture

DMS-79 small cell lung carcinoma cells [10, 11] were grown in Dulbecco's modified Eagle's medium supplemented with penicillin G (100 μ/ml), streptomycin sulfate (100 μg/ml), and 20% horse serum. Human genital skin fibroblasts were grown in minimum essential medium supplemented with antibiotics as above and 10% bovine calf serum. HepG2 human hepatoma cells were grown in Dulbecco's modified Eagle's medium supplemented with antibiotics as above plus 10% bovine calf serum. Stocks were maintained at 37°C in 75 cm^2 culture flasks in an atmosphere of 5% CO_2.

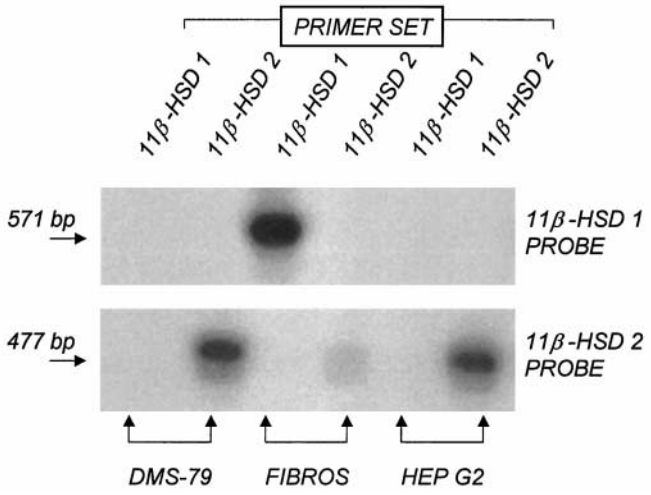

Fig. 1. 11β-HSD1 and 11β-HSD 2 mRNA expression in DMS-79 and control cells. DMS-79, fibroblast, and HepG2 cell mRNA was reversed transcribed and PCR amplified with primers shown in Table 1. The 11β-HSD1 primers amplify a 571 bp fragment of 11β-HSD1 cDNA; the 11β-HSD2 primers amplify a 477 bp fragment of 11β-HSD2 cDNA. Southern blots of RT–PCR products from each cell line are shown, with the primer sets specified immediately above each lane. Identical blots were examined using radiolabeled probes specific for 11-βHSD1 (top) and 11-βHSD1 (bottom).

RT–PCR

RT–PCR was performed with each of the three cell lines with primers designed to amplify 11β-HSD types 1 and 2 [12], as well as the ubiquitously expressed sodium–potassium ATPase as a control [12] (Table 1). Total RNA was purified from each cell line using a commercial kit (RNeasy Total RNA Kit, QIAGEN, Chatsworth, CA). Reverse transcription and PCR were performed using GeneAmp RNA PCR Kit (Perkin-Elmer, Branchburg, NJ). For each reaction a lower reagent mix was sealed below a wax layer (Ampliwax PCR Gem 100, Perkin-Elmer); this mix consisted of 2 mM $MgCl_2$, 1 × buffer, 0.3 μM 5′-sense primer, and the kit's DNA polymerase (2.5 U/reaction). Tubes were heated at 80°C for 5 min and cooled to 25°C for 1 min to seal the wax layer. The reverse transcription reagents were layered above the wax layer. This upper reagent layer consisted of 5 mM $MgCl_2$, 1 × buffer, 1 mM each dNTP, 20 U RNase inhibitor, 50 U Moloney murine leukemia virus reverse transcriptase, 0.3 μM 3′-antisense primer, and 10 μg RNA (total volume 100 μl). Tubes were heated to 42°C for 15 min (for reverse transcription), then to 95°C for 10 min (for inactivation of reverse transcriptase and melting of the wax to allow upper and lower reagents to mix). Samples were then subjected to 35 cycles of PCR (94°C for 1 min, 47°C for 1 min, and 72°C for 2 min) and a final 10 min cycle at 72°C.

In addition to total RNA from each cell line, positive controls were carried out using 1 ng of a cDNA clone for 11β-HSD1 or 11β-HSD2 as template.

Negative controls with each set of primers were run as above with the omission of the RNA. The 11β-HSD1 cDNA clone is the gift of Dr Perrin White (University of Texas Southwestern Medical Center, Dallas); the 11β-HSD2 cDNA was the gift of Dr Zygmunt Krozowksi (Baker Institute, Prahran, Victoria, Australia).

Southern blot analysis

Southern blots were prepared using 1–2 μl of the 100 μl total RT–PCR reaction. The DNA was resolved on a 1.2% agarose gel, transferred to nylon membrane (Nytran, Schleicher and Schull, Keene, NH) by capillary transfer, and immobilized by UV cross-linking (UV Stratalinker 1800, Stratagene, La Jolla, CA). Pre-hybridization was performed in 6 × SSPE, 10 × Denhardt's, 0.5% SDS, and 100 μg/ml salmon sperm (Sigma, St. Louis, MO), followed by hybridization at 42°C with 6 × SSPE, 50% formamide, 0.5% SDS, 10% dextran sulfate, and 50 μg/ml salmon sperm. An 11β-HSD1 probe was made by digestion of the plasmid with *Hind III* and *Bam*, followed by purification of the 1375 bp insert with the QIAEX II Gel Extraction Kit (QIAGEN). The 11β-HSD2 probe was made by purification of the 1900 bp *Xho 1*/*Hind III* fragment as above. The probes were radiolabeled with [α^{32}P]dATP by the random primers method using a commercial kit (Prime-It II, Stratagene). Nylon filters were washed at a final stringency of 0.1 × SSPE and 0.1% SDS at 55°C.

Enzyme assays for 11β-HSD activity

Cells. Adherent cells (fibroblasts and HepG2 cells) were plated (5×10^6 cells per 28.2 cm^2 well (Flow Laboratories, McLean, Virginia) one day prior to the experiment. Immediately prior to the assay, the medium was aspirated and the cells were washed with PBS. The non-adherent DMS-79 cells were removed from medium and washed with PBS just prior to each assay, then resuspended in MEM and added to 28.2 cm^2 wells at 5×10^6 cells per well. For each cell type in the assay, duplicate wells were also plated and harvested for quantization of protein.

Assay. 11β-HSD activity was quantized by measuring the conversion of radiolabeled 11-hydroxysteroids to the corresponding keto form using a whole-cell assay. Stock solutions of hydrocortisone, dexamethasone and glycyrrhetinic acid were dissolved in MEM without serum. Tracer amounts of [1,2-^{3}H(N)]hydrocortisone (NEN, Boston, MA) and varying amounts of non-radioactive cortisol (Sigma) were added to the cells for final hydrocortisone concentrations ranging from 4 to 200 nM. DMS-79 cells were also assayed with [1,2,4-^{3}H]dexamethasone (Amersham Life Science Inc., Cleveland, OH) at 20 nm. In addition, 18β-glycyrrhetinic acid (Aldrich, Milwaukee, WI) was added to assays where indicated. Cells were incubated with steroid substrates for 60 min at 37°C in an atmosphere of 5% CO_2. The reaction was terminated by the addition of 1 ml ethylene chloride.

Chromatography. Steroids were extracted with ethylene chloride, and the organic layer was separated and dried after the addition of non-radioactive steroid markers (Sigma). Samples were resuspended in chloroform and spotted on thin layer chromatography (TLC) plates (Silica-gel coated plastic plates with fluorescent indicator UV_{254}, Brinkmann, Westbury, NY). The steroids were separated by TLC in a solvent system of chloroform:methanol 90:10 (for hydrocortisone) and chloroform:ethanol 90:10 (for dexamethasone). Plates were sprayed with phosphomolybdic acid for visualization of steroid markers. Areas of the plate corresponding to visualized non-radioactive standards were cut out an placed in vials with 8 ml scintillation cocktail (Ecolite, ICN Pharmaceuticals, Costa Mesa, CA) and counted 48–72 h later.

Data analysis. At the time of each individual experiment, an equal volume of the solution of radiolabeled steroid in MEM was counted to obtain total counts and correct for recovery. Corrected counts were converted to molar equivalents based on adjusted specific activity of the isotope. Rates were calculated as pmol/h/mg protein.

RESULTS

RT–PCR

Total RNA from DMS-79 cells, as well as normal fibroblasts and the hepatoma cell line HepG2, was used as a template for RT–PCR using primers designed to amplify 11β-HSD types 1 and 2 [12]. The ubiquitously expressed Na–K ATPase was amplified from all three cell lines as a positive control. Using primers specific for 11β-HSD1, a fragment of the expected 571 bp was amplified only from normal fibroblasts. Using primers specific for 11β-HSD2, the expected 477 bp fragment was seen using RNA from DMS-79 and HepG2. The findings were confirmed by performing Southern blots of the PCR products and using radiolabeled probes corresponding to 11β-HSD types 1 and 2 (Fig. 1). The products from DMS-79 and HepG2 hybridized strongly to the type 2 probe, while the type 1 probe hybridized only to the product from fibroblasts.

β-HSD assays

Initial studies were performed to verify appropriate reaction conditions (data not shown). Linearity of product formation with respect to cell number (0.5–10×10^6) and time (10–90 min) was demonstrated. Also, during initial studies with each cell line the entire TLC lane was cut into 1 cm segments and

Table 2. Kinetic data for 11β-HSD activity in cell lines

	K_m for cortisol (nM)	V_{max} (pmol/mg protein/h)
DMS-79	26.1 ± 9.0	57.0 ± 5.9
HepG2	26.2 ± 11.8	25.8 ± 3.5
Fibroblasts	652	87.4

counted, showing a single reaction product migrating with authentic cortisone standard (data not shown).

Rates of cortisone production by DMS-79 cells, HepG2 hepatoma cells, and normal human fibroblasts were measured at concentrations of cortisol ranging from 4 to 200 nM (Fig. 2; Table 2). As significant portions of substrate were converted to product, the integrated form of the Henri–Michaelis–Menten was used to calculate the Michaelis constant for DMS-79 and HepG2. DMS-79 cells and HepG2 cells exhibited enzyme activities with similar K_m for cortisol –approximately 26 nM. The enzyme velocity in DMS-79 cells was about twice that observed in HepG2 cells (57.0 ± 5.9 vs 25.8 ± 3.5 pmol/mg protein/h). These data are consistent with expression of 11β-HSD2, the high affinity isoform of the enzyme. Since our assays were carried out in intact cells, we have not formally demonstrated the cofactor dependence of the observed 11β-HSD activity. The data observed in fibroblasts are suggestive of first-order kinetics, where [S] is much less than K_m. This is consistent with RT–PCR results showing expression of the type 1 isoform, with a K_m in the micromolar range.

The ability to metabolize dexamethasone is characteristic of 11β-HSD2; therefore conversion of dexamethasone was studied in DMS-79 cells. In triplicate reactions, assays were performed with 20 nM radiolabeled dexamethasone. Each TLC lane was cut into 1 cm sections, and radioactivity counted. A single peak was observed to migrate just ahead of the authentic dexamethasone standard (Fig. 3). This peak represents a mean conversion of 42% to the 11-keto form of dexamethasone.

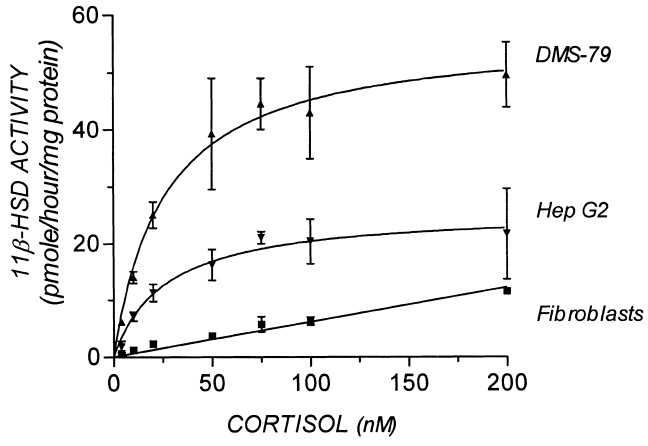

Fig. 2. 11β-HSD enzymatic activity in DMS-79 and control cells. DMS-79, fibroblast, and HepG2 cells were assayed for 11βHSD activity using cortisol as substrate. Rate vs substrate curves are shown for each cell line. Each point is the mean (±SEM) for three replicate experiments.

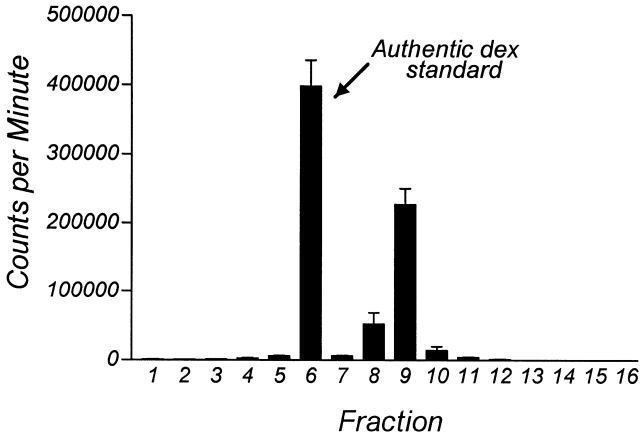

Fig. 3. Metabolism of dexamethasone in DMS-79 cells. DMS-79 cells were incubated with 20 nM [^{3}H] dexamethasone as described. Steroids were extracted and the samples run on thin layer chromatography plates. The lanes were cut into one centimeter sections beginning at the origin (fraction 1), the steroids eluted in scintillation cocktail, and counted for radioactivity. Fraction 6 corresponds to the mobility of the dexamethasone standard added for visualization. Data are shown as mean ± SEM for three independent experiments.

11β-HSD activity is inhibited by glycyrrhetinic acid in DMS-79 cells. With cortisol as substrate at a concentration of 20 nM, the addition of glycyrrhetinic acid resulted in a dose-dependent inhibition of enzyme activity to as low as 7% of baseline values (Fig. 4). Dexamethasone metabolism in DMS-79 cells was also inhibited by the addition of 10 μM glycyrrhetinic to 12% of baseline (Fig. 4, inset).

DISCUSSION

The apparent glucocorticoid resistance exhibited by ACTH-producing neoplasms has been exploited for clinical diagnosis for over thirty years, although the molecular basis for this phenomenon has remained unknown. While supraphysiologic doses of glucocorticoids (dexamethasone at 0.5 mg q 6 h [4]) suppress normal pituitary function, they fail to inhibit ACTH production from pituitary corticotroph tumors. However, higher doses of dexamethasone (2.0 mg q 6 h) can suppress ACTH in the majority of cases of pituitary Cushing's [13]. This relative glucocorticoid resistance of ACTH producing pituitary neoplasms had been well-established when it was recognized that non-pituitary neoplasms could also produce ACTH [3]. Such 'ectopic' sources of ACTH were found, in general, to be characterized by more profound glucocorticoid resistance, so that no amount of glucocorticoid could suppress ACTH secretion. A subset of ectopic ACTH-producing tumors, particularly bronchial carcinoids [14] exhibit a relative gluco-

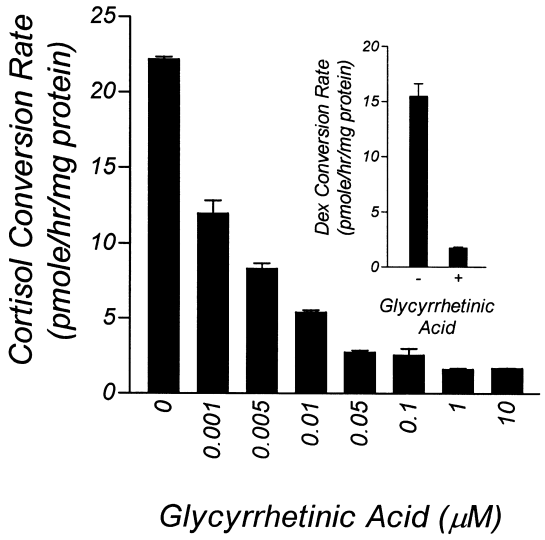

Fig. 4. Glycyrrhetinic acid inhibition of 11β-HSD activity in DMS-79 cells. The rate of conversion of cortisol to cortisone is shown as a function of the added concentration of the known 11β-HSD inhibitor, glycyrrhetinic acid. *Inset*: Inhibition of dexamethasone metabolism in DMS-79 cells by the 10 μM glycyrrhetinic acid. Reaction rates for the conversion of dexamethasone by DMS-79 cells were calculated as described. Experiments were carried out in the absence (–) and in the presence (+) of glycyrrhetinic acid.

corticoid resistance similar to that observed in the case of ACTH-producing pituitary neoplasms.

A number of possible mechanisms might be responsible for the varying degrees of glucocorticoid resistance observed in different types of ACTH-producing tumors. Defects in glucocorticoid receptor structure and function have been identified now in a number of cell lines established from non-pituitary ACTH-producing tumors [5, 6, 15, 16]. These defects have been, in each instance documented to date, of such a nature as to confer absolute glucocorticoid resistance on these tumors. Other mechanisms might underlie the relative glucocorticoid resistance observed in pituitary tumors secreting ACTH and in some 'ectopic' ACTH-producing tumors. Expression of ligand-excluding systems such as the *mdr* gene product or enzymes such as 11β-HSD are examples of such potential mechanisms.

11β-HSD2, the tissue-specific, high affinity, NAD^+-dependent form of 11β-HSD, is of central physiologic importance in the action of mineralocorticoids [17]. Since the mineralocorticoid receptor lacks the capacity to discriminate between glucocorticoid and mineralocorticoid signals, the co-expression of 11β-HSD is necessary to exclude glucocorticoid ligands from the mineralocorticoid receptor. The characteristics of 11β-HSD2 (high affinity for natural glucocorticoids as well as for dexamethasone) were reminiscent of the clinical data demonstrating that ACTH-producing tumors are resistant to both natural (cortisol) and synthetic (dexamethasone) glucocorticoids. Whether expression of 11β-HSD2 might underlie some such instances of apparent glucocorticoid resistance is unknown.

We found expression of 11β-HSD2 in DMS-79 cells derived from an ACTH-producing small cell lung cancer. Expression of 11β-HSD2 in human tissues has been carefully surveyed using the RT/PCR approach on which we have based our RNA analysis [12]. While 11β-HSD1 is widely distributed in liver, placenta, lung, spleen, brain, kidney, colon, and salivary gland, 11β-HSD2 enzyme expression was found to be limited to kidney, colon, salivary gland, and placenta. Lung, liver, and brain did not appear to express the type 2 enzyme mRNA. Our findings in DMS-79 cells at the mRNA level are corroborated by enzymatic studies that confirm expression of an enzyme activity with kinetics, substrate utilization and inhibitor sensitivities characteristic of 11β-HSD2.

Previous studies in the DMS-79 cell line have revealed expression of an abnormal glucocorticoid receptor mRNA transcript encoding a GR lacking the steroid binding domain [5, 6]. Only minimal amounts of normal GR are detectable at the mRNA level by northern blot and at the protein level by affinity labeling experiments with radiolabeled dexamethasone mesylate [5]. Thus, with inhibition of 11β-HSD2 by glycyrrhetinic acid we were still unable to restore normal glucocorticoid binding or glucocorticoid signaling (data not shown). While 11β-HSD2 expression is therefore, not the reason for the profound glucocorticoid resistance of DMS-79 cells, our observation does raise the possibility that expression of the enzyme might confer some degree of glucocorticoid resistance on some ACTH- producing tumors with otherwise intact glucocorticoid signaling pathways. Primary specimens of such tumors will need to be examined and correlated with clinical physiologic data from the affected patients to rigorously address the question of whether tumor expression of 11β-HSD2 might confer local glucocorticoid resistance and impaired negative feedback of glucocorticoids on ACTH production.

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