Dexamethasone does not exert direct intracellular feedback on steroidogenesis in human adrenal NCI-H295A cells

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Abstract

Experimental therapy of fetuses affected with congenital adrenal hyperplasia (CAH) has been reported by administering dexamethasone (Dex) to pregnant women at risk for carrying a CAH fetus. Such prenatal therapy can almost wholly eliminate virilization of the external genitalia of affected female fetuses, but only when treatment is started before 9 weeks of gestation. As it is not known whether the hypothalamic–pituitary–adrenal axis is functional at this time, and as the minimal effective doses of Dex are substantially supraphysiologic for the fetus, the mechanism of action of prenatal Dex treatment has been unclear.

To assess the possibility that Dex might act directly on the adrenal, we used human adrenocortical NCI-H295A cells, an established model of the human fetal adrenal. Short term (6 h) incubation of these cells with Dex decreased synthesis of 11-deoxycortisol and 17 α -hydroxyprogesterone and increased synthesis of deoxycorticosterone (DOC), but only at very high con-

centrations of Dex ($\geq 10 \mu\text{M}$) that affect P450c17 (17 α -hydroxylase/17,20 lyase) as a competitive inhibitor; no effects were seen at lower concentrations corresponding to those used in prenatal treatment. When NCI-H295A cells were treated with up to 100 μM Dex for 72 h, dot-blot analysis showed no changes in the abundance of the mRNAs for P450scc (cholesterol side-chain cleavage enzyme), P450c17, or 3 β -hydroxysteroid dehydrogenase II (3 β HSDII). When NCI-H295A cells were transfected with promoter/reporter constructs for the human genes for P450scc and P450c17, 24-h treatment with Dex at doses up to 100 μM had no effect on the transcription of these constructs. Because the regulation of steroidogenic processes in NCI-H295A cells closely resembles such regulation in the adrenal, we suggest that Dex may not act by an intra-adrenal mechanism in the experimental prenatal treatment of CAH.

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Introduction

Mutations in the gene for steroid 21-hydroxylase cause over 90% of cases of congenital adrenal hyperplasia (CAH) (Miller 1994, White & Speiser 2000), which affects about 1 in 14 500 individuals (Therrell *et al.* 1998). In affected children and adults, decreased 21-hydroxylase activity impairs the conversion of 17 α -hydroxyprogesterone (17 OHP) to 11-deoxycortisol, causing glucocorticoid deficiency which, in turn, results in increased secretion of adrenocorticotropin (ACTH). Increased ACTH stimulates the steroidogenic steps prior to 21-hydroxylase resulting in overproduction of adrenal C-19 steroid precursors of androgens. The genitalia of affected 46XX genetic females can be severely virilized, suggesting that the same physiology occurs *in utero*. To suppress fetal ACTH and consequently ameliorate genital virilization of affected females, David and Forest (1984) and Evans *et al.* (1985) first proposed the administration of

glucocorticoids such as dexamethasone (Dex) early in pregnancy to women at risk for delivering an infant with CAH (based on having delivered a previous child with CAH). This approach has been refined and popularized by New and colleagues (Speiser *et al.* 1990, Mercado *et al.* 1995, New *et al.* 2001) and by Forest and colleagues (Forest *et al.* 1989, 1993, 1998). Experience with over 200 fetuses treated to term indicates that prenatal administration of Dex substantially ameliorates or eliminates the genital virilization of affected females, if the treatment is started less than 9 weeks following fertilization (Forest *et al.* 1998, New *et al.* 2001). However, this approach remains controversial (Seckl & Miller 1997, Miller 1998), and the worldwide pediatric endocrine community has agreed that it is not 'standard-of-care' and requires prospective protocols approved by local human experimentation committees (Joint LWPES/ESPE CAH Working Group 2002).

The mechanism of action of Dex in this experimental prenatal treatment of CAH has been unclear. Dex, rather

than cortisone or prednisone, is used because it is not an effective substrate for placental 11 β -hydroxysteroid dehydrogenase 2 (11 β HSD 2) (White *et al.* 1997) and hence is not inactivated by the placenta. The best clinical results are obtained when Dex treatment is started very early in gestation, at 6–8 weeks post conception, before the time when testicular androgens begin to virilize the external genitalia in normal male development at 6–14 weeks (Wilson *et al.* 1993). Although immunoreactive ACTH can first be detected in the fetal pituitary at about 8 weeks of gestation (Asa *et al.* 1986, 1988), adrenal development appears to be normal in first-trimester anencephalic fetuses, suggesting that early adrenal development may be independent of ACTH (Gray & Abramovich 1980). Furthermore, fetal concentrations of cortisol are normally about one-tenth of those of the mother (Partsch *et al.* 1991, Kari *et al.* 1996) and the typical doses of Dex (20 μ g/kg maternal weight) are about 3 to 6 times supraphysiological for the mother. Thus, if Dex freely crosses the placenta, the effective glucocorticoid dose delivered to the fetus may be up to 60 times supraphysiologic.

While the glucocorticoid feedback sensitivity of the fetal hypothalamic–pituitary axis is unknown, it has appeared that the high dosage requirements are consistent with a mechanism other than suppression of the hypothalamic–pituitary–adrenal (HPA) axis. One possibility is that Dex might affect the adrenal directly. If the adrenal is normally responsive to hypothetical ultra-short loop feedback by glucocorticoids, one might expect that high glucocorticoid concentrations in the range of those found within the adrenal, rather than those found within the circulation, would exert such an effect. A preliminary study in our laboratory in 1990 suggested that Dex may directly suppress the transcription of the gene for P450c17 (17 α -hydroxylase/17,20 lyase) (Brentano *et al.* 1990). Therefore we explored the possibility that high concentrations of Dex may exert a direct intra-adrenal ultra-short loop feedback on steroidogenesis.

Materials and Methods

Steroid production

The adherent NCI-H295A subline (Rodriguez *et al.* 1997) of human NCI-H295 cells (Gazdar *et al.* 1990, Staels *et al.* 1993) was grown to confluence in 6-well plates in RPMI medium supplemented with 2% FBS, penicillin (100 U/ml), 5 μ g/ml insulin, 5 μ g/ml transferrin and 5 ng/ml selenite, as described (Staels *et al.* 1993, Rodriguez *et al.* 1997). Concentrations of 0.1, 1.0, 10 and 100 μ M Dex (Sigma) in 50 μ l ethanol were added for 6 h, then 20 000 c.p.m. [14 C]pregnenolone or [14 C]progesterone (NEN Life Science, Boston, MA, USA) were added for 1 h. The medium (2 ml) was harvested and steroids were extracted with 8 ml methylene chloride:iso-octane

(1:1), dried under N₂, dissolved in 20 μ l methylene chloride, and analyzed by thin layer chromatography on Whatman PE Sil G/UV silica gel plates (Maidstone, Kent, UK) as described (Auchus *et al.* 1998). Chromatograms were developed with 3:1 chloroform:ethyl acetate to resolve 17-hydroxysteroids (Lin *et al.* 1991) or 300:20:1 methylene chloride:methanol:H₂O to resolve 17-deoxysteroids (Fardella *et al.* 1995) as described. The 14 C steroids were detected by phosphorimaging and identified by co-chromatography with a panel of labeled and unlabeled steroid standards.

RNA analysis

Total RNA was extracted from NCI-H295A using 0.5 ml TriReagent (Molecular Research Center, Cincinnati, OH, USA) (Chomczynski 1993) according to the manufacturer's instructions. Dot-blot analysis was performed as described (Voutilainen *et al.* 1986). RNA samples were denatured in 7.5% formaldehyde and 6 $\times$ SSC (0.9 M NaCl, 0.09 M sodium citrate) at 80 °C for 20 min and then blotted to nitrocellulose filters (Amersham). Filters were prehybridized in buffer containing 50% formamide, 6 $\times$ SSC, 0.1% ficoll, 0.1% BSA, 0.1% polyvinylpyrrolidone, 100 μ g/ml salmon sperm DNA and 100 μ g/ml yeast tRNA for 16 h at 42 °C before probe was added. Hybridization was performed for 4 h using labeled human cDNA for P450c17 (Chung *et al.* 1987), P450sc (cholesterol side chain cleavage enzyme) (Chung *et al.* 1986), or 3 β HSDII (3 β -hydroxysteroid dehydrogenase II) (Rheume *et al.* 1991). Probes were labeled with [α - 32 P]dCTP as described (Feinberg & Vogelstein 1984). The uniformity of the loading was tested using a labeled human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe generated by PCR. The filters were washed in 0.2% SSC, 0.1% SDS, 4 times at 50 °C and analyzed by phosphorimaging.

Transfection and dual luciferase reporter assay

Luciferase reporter constructs containing 2500 bp of the 5' flanking DNA of the P450c17 gene (Rodriguez *et al.* 1997) or 2327 bp of the 5' flanking region of the P450sc gene (Moore *et al.* 1990), were transfected into NCI-H295A cells using the calcium phosphate-DNA coprecipitation method (Gorman *et al.* 1982) as described (Rodriguez *et al.* 1997). For transfection, the RPMI medium was replaced by DMEM-H16 supplemented with 10% FBS and penicillin (10 U/ml) as described (Rodriguez *et al.* 1997). After 12 h, the medium was replaced with fresh medium containing various concentrations of Dex and incubated for 24 h. Cellular extracts were assessed for luciferase activity by the dual luciferase reporter assay system (Promega). Transfection efficiencies

were normalized by co-transfecting the cells with the pRL-CMV plasmid (Promega) expressing the *Renilla* luciferase gene driven by the CMV promoter.

Results

To determine whether dexamethasone exerts an intra-adrenal ultra-short loop feedback inhibition on steroidogenesis, we used human adrenal NCI-H295A cells as a model of the human fetal adrenal (Staels *et al.* 1993). These cells secrete the same steroids that are produced by the human adrenal (Gazdar *et al.* 1990), and express all the enzymes normally found in the human fetal adrenal gland (Staels *et al.* 1993). The regulation of steroid hormone synthesis, the regulation of the mRNAs for the steroidogenic enzymes and the transcriptional regulation of their cognate genes all respond in the physiologically expected fashions, providing excellent evidence that the regulation of steroidogenesis in these cells is a useful model for the regulation of steroidogenesis in the human fetal adrenal (Gazdar *et al.* 1990, Rainey *et al.* 1993, Staels *et al.* 1993, Rainey *et al.* 1994, Bird *et al.* 1995, Rodriguez *et al.* 1997, Bird *et al.* 1998, Wijesuriya *et al.* 1999, Lin *et al.* 2001). The potential action of Dex on the adrenal could conceivably be exerted at any of three levels. First, Dex might exert an action directly on steroidogenic enzymes, either as an inhibitor of enzymatic activity or by altering the stability of the enzymes themselves; secondly, Dex might alter the stability of the mRNAs encoding steroidogenic enzymes; thirdly, it might have a direct effect to suppress transcription of genes encoding steroidogenic enzymes.

Examining the conversion of [14 C]pregnenolone to downstream steroids in a short (6 h) incubation period provides a measure of the potential action of Dex on steroidogenic enzymes. Under basal conditions, NCI-H295A cells convert pregnenolone to the Δ^4 steroids, progesterone, 17 OHP, deoxycorticosterone (DOC), 11-deoxycortisol, and androstenedione, and secrete very little of the Δ^5 steroids, 17 α -hydroxypregnenolone (17 OHPreg) and dehydroepiandrosterone (DHEA) (Gazdar *et al.* 1990). Concentrations of Dex up to 1 μ M had no effect on steroidogenesis, but 10 μ M Dex decreased secretion of 11-deoxycortisol and 17 OHP and increased secretion of DOC, suggesting an inhibitory effect on 17 α -hydroxylase activity (Fig. 1). When the cells were incubated with [14 C]progesterone, thus providing a Δ^4 steroid precursor, 10 μ M Dex again inhibited 17 α -hydroxylase activity, increasing secretion of DOC and decreasing secretion of 17 OHP and 11-deoxycortisol (Fig. 2). Thus Dex inhibited 17 α -hydroxylase activity, but only at the very high concentrations that are known to act as a competitive inhibitor of P450c17 (Lee *et al.* 1999).

To determine whether Dex exerts an effect on the abundance of steroidogenic enzyme mRNAs, we measured the abundance of the mRNAs for P450scc (the

Figure 1 Metabolic conversion of pregnenolone in NCI-H295A cells incubated with Dex. Cells were incubated with [14 C]pregnenolone and with the indicated concentrations of Dex for 6 h, and the generated metabolites were analyzed in two different chromatographic systems: chloroform-ethyl acetate 3:1 (A) and methylene chloride-methanol-H₂O, 300:20:1 (B). The identity of the identified radioactive steroids was confirmed by co-chromatography with standards for pregnenolone (Preg), progesterone (Prog), 17 OHP, 17 OHPreg, androstenedione (Andro), DOC, 11-deoxycortisol (11 Deoxycort), corticosterone, aldosterone, and hydrocortisone.

quantitative regulator of steroidogenesis), P450c17 (the qualitative regulator of steroidogenesis) and 3 β HSDII. Incubating NCI-H295A cells with 0.1 to 100 μ M Dex for 72 h did not change the abundance of any of these mRNAs (Fig. 3). As a potential effect of Dex might be to inhibit tropic stimulation, we again incubated NCI-H295A cells with 0.1 to 100 μ M Dex for 72 h, but added 1 mM 8Br-cAMP during the last 24 h. The cAMP treatment increased P450scc mRNA eight- to ninefold and increased P450c17 and 3 β HSD mRNA about two-fold, but this action of cAMP was not influenced by Dex (Fig. 4). The substantially greater response of P450scc mRNA to cAMP compared with P450c17 mRNA is consistent with prior observations in NCI-H295 cells (Staels *et al.* 1993) and in primary cultures of human fetal adrenal cells (Di Blasio *et al.* 1987).

Finally, to determine whether Dex exerts a direct effect on transcription of genes encoding steroidogenic enzymes,

Figure 2 Metabolic conversion of progesterone in NCI-H295A cells incubated with Dex. Cells were incubated with [^{14}C]progesterone and with the indicated concentrations of Dex for 6 h, and the metabolites generated were analyzed by chromatography in methylene chloride-methanol- H_2O , 300:20:1. (A) Phosphorimage of a typical experiment. (B) Quantification of the DOC (black bars), 17 OHP (grey bars) and 11-deoxycortisol (white bars) identified in panel A. Data are expressed as percentage of the added counts converted to the indicated steroid in a single, representative experiment. Prog, progesterone; Andro, androstenedione; 11 Deoxycort, 11-deoxycortisol.

we transfected NCI-H295A cells with promoter/reporter constructs containing 2.3 kb DNA from the human P450scc promoter or 2.5 kb DNA from the human P450c17 promoter. These DNA segments contain all of the elements known to participate in the regulation of these key genes (Brentano *et al.* 1990, Moore *et al.* 1990, Rodriguez *et al.* 1997, Lin *et al.* 2001). No concentration of Dex up to 100 μM had a detectable effect on the transcription of these constructs during a standard (Lin *et al.* 2001) 24-h incubation (Fig. 5). Thus, although high concentrations of Dex inhibited 17 α -hydroxylase activity,

Figure 3 Effect of Dex on the mRNAs for steroidogenic enzymes. Dot-blot of total RNA from NCI-H295A cells were probed with cDNAs for human P450scc (black bars), P450c17 (grey bars), and 3BHSD (white bars), analyzed by phosphorimaging. The amount of each mRNA in cells treated for 72 h with each concentration of Dex was expressed as a percentage of that value in untreated cells (without Dex). Data are means $\pm$ S.D. of three independent experiments, each performed in duplicate. There was no difference ($P > 0.05$) in the abundances of any of these mRNAs when comparing the untreated cells with cells treated with any of the indicated concentrations of Dex.

they had no detectable affect on the transcription of genes encoding steroidogenic enzymes, or the half-life of their encoded mRNAs.

Discussion

In order for prenatally administered Dex to suppress the genital virilization of CAH female fetuses, it must be administered at very high doses very early in gestation, suggesting Dex may act by a mechanism other than suppressing the HPA axis.

An early report indicated that intracellular steroids exert regulatory effects on the activity of the steroidogenic enzymes in adrenals, testis and ovaries, thus regulating the pattern and the rate of steroid production (Gower & Cooke 1983). In bovine and guinea pig adrenals, androstenedione can inhibit 21-hydroxylase activity, increasing C-19 steroid synthesis and inhibiting glucocorticoid production (Hornsby 1982, Belanger *et al.* 1995). However, the potential effects of steroids on their own synthesis in adrenocortical cells is not clear. ACTH induces accumulation of mRNAs for P450scc and P450c17 in primary cultures of human fetal adrenal cells (Di Blasio *et al.* 1987), and Dex inhibited the ACTH-induced accumulation of P450scc and P450c17 mRNAs in cultured bovine adrenocortical cells (Trzeciak *et al.* 1993), but Dex enhanced ACTH induction of corticosteroid production in ovine adrenocortical cells (Darbeida & Durand 1988),

Figure 4 Effect of Dex and cAMP on the mRNAs for steroidogenic enzymes. Dot-blots of total RNA from NCI-H295A cells were probed with cDNAs for human P450scc (black bars), P450c17 (grey bars), and 3βHSD (white bars), and analyzed by phosphorimaging. Cells were incubated with the indicated concentrations of Dex for 72 h as in Fig. 3, but 1 mM 8Br-cAMP was added during the final 24 h incubation. The amount of each mRNA in cells treated with cAMP was expressed as a percentage of the corresponding value in untreated cells. Data are means $\pm$ S.D. of three independent experiments, each performed in duplicate. Each mRNA increased significantly ($P < 0.05$) in cells treated with cAMP, but there was no difference ($P > 0.05$) in the abundances of any of these mRNAs due to the presence of Dex.

Figure 5 Effect of Dex on P450scc and P450c17 promoter activity. NCI-H295A cells transfected with promoter/reporter constructs containing 2327 bp of the 5' flanking region of the P450scc gene (black bars) or 2500 bp of the 5' flanking DNA of the P450c17 gene (white bars) were treated with the indicated concentrations of Dex for 24 h and luciferase activity was measured and expressed as a percentage of the value in untreated cells (without Dex). Data are means $\pm$ S.D. of three independent experiments, each performed in triplicate. There was no effect ($P > 0.05$) on the promoter activity of P450scc or P450c17 at any concentration of Dex tested.

and phorbol ester induction of 3β-hydroxysteroid dehydrogenase in NCI-H295R cells (Feltus *et al.* 2002). Thus, we investigated the potential role of Dex as a potential regulator of fetal adrenal steroidogenesis.

Human adrenal NCI-H295A cells are the best available model of the human fetal adrenal. They produce all of the steroids and express all of the enzymes of human adrenal steroidogenesis, and regulate the genes encoding these enzymes in a manner similar to that of human fetal adrenal cells (Gazdar *et al.* 1990, Staels *et al.* 1993, Rainey *et al.* 1993, 1994, Bird *et al.* 1995, Rodriguez *et al.* 1997, Bird *et al.* 1998, Wijesuriya *et al.* 1999, Lin *et al.* 2001). They also express insulin-like growth factor-II (Staels *et al.* 1993), a marker of the fetal but not the adult adrenal (Voutilainen & Miller 1988), and express functional glucocorticoid receptor (Fassnacht *et al.* 2000, Feltus *et al.* 2002). Nevertheless, since this is a transformed cell line, there may be unknown aspects of the regulation of steroidogenesis that differ between NCI-H295A cells and the human fetal adrenal gland *in vivo*.

In human adrenal replacement therapy, less than 0.2 mg Dex per m² of body surface area appears to be physiologic (Rivkees & Crawford 2000), and estimations of the biopotency of Dex based on its ability to suppress adrenal C-19 steroid secretion (Rivkees & Crawford 2000) and growth (Styne *et al.* 1977) suggest that Dex is 80 times more potent than hydrocortisone, rather than the generally-cited factor of 30 based on anti-inflammatory effects. The normal secretory rate of cortisol is 6–7 mg/m²/day (Linder *et al.* 1990, Esteban *et al.* 1991, Kerrigan *et al.* 1993), which would thus translate to a Dex equivalent of about 0.1 mg/m². The higher therapeutic potency of Dex is related to its longer half-life than cortisol, its poor binding to corticosteroid binding globulin and to its sevenfold higher binding affinity to the glucocorticoid receptor (Manz *et al.* 1983, Derendorf *et al.* 1993). However, the typical dose of Dex used in the prenatal treatment of CAH is 20 μg/kg/day (Forest *et al.* 1989, Speiser *et al.* 1990, Forest *et al.* 1993, Mercado *et al.* 1995, Forest *et al.* 1998, New *et al.* 2001). Thus, in a 60 kg woman having a body surface area of 1.2 m², the physiologic replacement dose of Dex is probably less than 0.2 mg, but the dose used in the prenatal treatment of CAH is 1.2 mg. This is about 6 times supraphysiological for the mother, and, as fetal cortisol concentrations are normally one-tenth those in the mother (Partsch *et al.* 1991, Kari *et al.* 1996), about 60 times supraphysiological for the fetus. This suggested that the action of Dex in experimental prenatal therapy might not be on the HPA axis.

The typical intra-adrenal concentrations of cortisol late in gestation and in newborns are about 3.7 to 7.4 μM and up to 47.6 μM in adults (Dickerman *et al.* 1984), whereas the circulating concentrations are less than 0.1 μM in the fetus (Partsch *et al.* 1991, Kari *et al.* 1996) and 0.1 to 0.7 μM in adults. Thus a hypothetical intra-adrenal

ultra-short loop feedback mechanism might be expected to act at concentrations 50 to 100 times higher than those found in the circulation, i.e. at $\approx 50 \mu\text{M}$ cortisol, which would be equivalent to 0.5 to 1 μM Dex. However, the only action of Dex we observed in concentrations up to 100 μM was a decrease in 17 α -hydroxylase activity at 10–100 μM Dex, which is consistent with the known action of Dex as a competitive inhibitor of P450c17 with a K_i of 87 μM (Lee *et al.* 1999). Such high concentrations of Dex are far beyond the concentrations that might approximate the intra-adrenal concentration of cortisol and far beyond those achieved by current prenatal protocols.

Thus our data suggest that the Dex used in experimental prenatal treatment of CAH does not work by an intra-adrenal mechanism. The mechanism by which Dex suppresses androgen production during prenatal treatment of CAH remains unclear.

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