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THE EFFECTS OF PROGESTERONE, PREGNENOLONE, ESTRIOL, ACTH AND hCG ON STEROID SECRETION OF CULTURED HUMAN FETAL ADRENALS

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SUMMARY

Endogenous steroid secretion and the conversion of exogenous pregnenolone and progesterone were studied in tissue culture of human mid-term fetal adrenals. Addition of pregnenolone on the first day of cultivation caused an increase in DHAS (dehydroepiandrosterone sulphate) and pregnenolone sulphate secretion during the first cultivation days, but no significant increase in cortisol production was noted. Progesterone at the same stage of cultivation was effectively converted into cortisol indicating that human fetal adrenals in spite of the lack of 3β-HSD (3β-OH-steroid dehydrogenase) are capable of synthesizing efficiently cortisol using exogenous (*in vivo* placental) progesterone as a substrate. During later stages of cultivation pregnenolone was converted into pregnenolone sulphate, but not into DHAS, indicating that sulphokinase activity is maintained in cultured adrenal cells. Estriol and estradiol-17β inhibited steroidogenesis in ACTH-stimulated tissue culture, and the inhibition step seemed to be 3β-HSD-step. The demanded concentrations of estrogens for significant inhibition were, however, unphysiological. Estriol-3-sulphate and DHAS did not inhibit steroidogenesis. hCG did not stimulate DHA or DHAS production, neither did it modify the stimulatory effect of ACTH towards these estrogen precursors.

INTRODUCTION

The regulation of the differentiation and secretion of human fetal adrenals forms a very complicated question. The secretory pattern of fetal adrenal cortex has been well described [e.g. 1–4], but the links between fetal and permanent zones have remained unclear. In studying human fetal adrenals in tissue culture [5, 6], it has been found that permanent zone cells are able to differentiate into adult fasciculata-like cells and to show the adult secretory pattern under influence of ACTH. Fetal zone cells rapidly disappear from cultures during the first days of cultivation, which indicates that these cells are highly differentiated and dependent on some trophic hormone(s), (one of) which may be ACTH. The cultivated fetal zone cells maintain the fetal secretory pattern without any sign of activation of the 3β-OH-steroid dehydrogenase delta-5-delta-4 isomerase (3β-HSD) system.

In the present work the activity of this 3β-HSD and the steroid forming capacity of human fetal adrenals are further studied in tissue culture by using first exogenous pregnenolone and progesterone as substrates. Secondly, based on the evidence of Goldman[7] of the inhibitory effect of estrogens on 3β-HSD in rat adrenals, the influence of some estrogens on human fetal adrenal steroidogenesis is tested in tissue culture. Thirdly the effect of hCG on steroidogenesis is tested in cultured fetal adrenals, because this hormone has been claimed to be the stimulator of DHA(S) production in fetal adrenals [8, 9].

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EXPERIMENTAL

Tissue culture experiments

Human fetal adrenal tissue, obtained from abortions induced for sociomedical reasons, was cultured in the manner described by Kahri[10]. The CR-length of the fetuses used varied from 6.5 to 15.5 cm corresponding to a gestational age of 11–18 weeks [11]. The tissue culture medium (5 ml/dish) was routinely replaced every sixth day and consisted of 50% Melnick A (0.5% lactalbumin hydrolysate in Hanks' balanced salt solution (BSS)), 25% Eagle's Minimum Essential Medium (both from Pharmaceutical Manufacture Orion Oy, Finland), and 25% heat-inactivated newborn calf serum (Gibco, United Kingdom). The amount of adrenal tissue per culture dish was 10–20 mg; equal amounts of adrenal tissue were explanted on parallel dishes in each culture group. Because the main purpose of the experiments was to measure the relative secretion of certain steroids in different experimental conditions, it was not essential to know the exact amount of tissue per dish. The cultures were studied by phase contrast microscopy for cell viability and experimental effects. When the effect of pregnenolone and progesterone was tested on adrenal steroidogenesis, these steroids (pregnenolone: E. Merck, Darmstedt, West Germany; progesterone: Steraloids, N.Y., U.S.A.) were added as a single dose into the tissue culture medium in ethanol

diluted with Hanks' BSS. The same small amount of ethanol, diluted with Hanks' BSS, was added to the control cultures. When the effects of estriol, estradiol-17β, estriol-3-sulphate or dehydroepiandrosterone sulphate (DHAS) were tested (all from Sigma Chemicals, St. Louis, U.S.A.), these steroids were added as a single dose in ethanol diluted with Hanks' BSS after changing the tissue culture medium on the sixth day of cultivation. ACTH stimulation (Synacthen, Ciba-Geigy Limited, Basel, Switzerland; 0.2 μg/ml = 0.02 IU/ml of tissue culture medium/day) was started on the same day and continued for 6 days. The dose of ACTH used causes maximal stimulation of steroidogenesis in the present system. hCG (human chorionic gonadotrophin; Pregnyl, Organon, Holland) was added at the concentration of 1 IU/ml/day. After experiments the tissue culture media were stored at -20°C until analysed.

Steroid determinations from culture media

The steroid determinations from culture media were made by a method slightly modified from that described by Salmenperä and Kahri[12]. The method used is described in detail elsewhere [13]. Only a brief description is given here. After addition of radioactive steroids as recovery tracers into the media, dichloromethane extraction was performed. The dried extracts were partitioned between 1 vol. of 80% ethanol and 4 vol. of cyclohexane. The samples were then fractionated on a 0.5 g Sephadex LH-20 column. The "androgen fraction" from the Sephadex column was further purified on a 0.15 g Hi-Flosil column. Cortisol, corticosterone, 11-deoxycortisol, DHA and androstenedione (4-androstene-3,17-dione) were quantitated as their O-methyloxime-trimethylsilyl derivatives with gas liquid chromatography using 1% SE-30 packed columns. A flame ionization detector was used and the responses were recorded with a digital integrator. For analysing sulphate conjugates the "water" residue of the primary dichloromethane extraction was used. The sulphate conjugates were solvolysed essentially as described by others [14, 15]. The solvolysed steroids were purified on a similar Hi-Flosil column as the free "androgens". After column chromatography the solvolysed and free steroids were quantitated by gas liquid chromatography. The methodological losses were corrected according to the recovery indicators. The precision of the method in the concentrations measured here was of the order of 2–17% for different steroids. Student's t-test (two sided) was used for testing the statistical significance of the results.

RESULTS

The effects of exogenous pregnenolone and progesterone on steroidogenesis

The endogenous secretion of human fetal adrenals in tissue culture during the first 6 days of cultivation can be seen in the first column in Table 1. The other columns show the effect of added pregnenolone and progesterone on steroidogenesis at the same time. At this stage of cultivation both fetal and permanent zone cells were present in the culture. Table 1 shows that very little free steroids were secreted endogenously in respect of the large amount of sulphate-conjugated steroids of the delta-5 series. Addition of pregnenolone caused a clear increase in sulphate conjugates, but only a slight increase in free steroids (especially of the delta-4-keto-3 series). The effect of added progesterone was quite different from that of pregnenolone: the free steroids of the delta-4-keto-3 series clearly increased, a substantial part of these being cortisol. No essential effect on DHAS was noted, pregnenolone sulphate increased slightly.

Table 2 shows the effect of pregnenolone, progesterone and ACTH on steroidogenesis during the 12–18th days of cultivation (when the cells are merely of the permanent cortex type). Endogenous secretion was lower than during the first cultivation days. Addition of pregnenolone increased only pregnenolone sulphate, but not DHAS. Progesterone addition caused only a very slight increase in the delta-4-keto-3 steroids, mainly corticosterone (not cortisol as during the first cultivation days). The last column of the table shows the clear stimulatory effect of ACTH on steroidogenesis.

The effect of estrogens on steroidogenesis

Figure 1 shows the effect of estriol on ACTH-induced steroidogenesis in human fetal adrenal culture. Estriol caused a clear decrease in cortisol (68.8%; $P = 0.0015$), corticosterone (64.3%; $P = 0.018$) and 11-deoxycortisol (46.5%; $P = 0.052$)

Table 1. Endogenous secretion and the effects of exogenous pregnenolone and progesterone on steroidogenesis in tissue culture of human fetal adrenals during the first six days of cultivation

	Control	Pregnenolone	Progesterone
Cortisol	24.6 ± 5.0	58.4 ± 6.8	1600 ± 225
11-deoxycortisol	<10	<10	101 ± 5
Corticosterone	<10	<10	257 ± 16
Dehydroepiandrosterone	22.5 ± 8.8	47.1 ± 20.6	18.6 ± 7.3
Androstenedione	<10	<10	27.6 ± 0.7
DHAS (dehydroepiandrosterone sulphate)	2290 ± 450	2670 ± 331	1890 ± 71
Pregnenolone sulphate	434 ± 21	1420 ± 142	851 ± 38

Pregnenolone and progesterone were added as a single dose (20 μg) on the first day of cultivation. Means ±S.E.M. of three cultures in each group are indicated. The units are ng/ml of tissue culture medium.

Table 2. Endogenous secretion and the effects of exogenous pregnenolone, progesterone and ACTH on steroidogenesis in tissue culture of human fetal adrenals during the 12–18th days of cultivation

	Control	Pregnenolone	Progesterone	ACTH
Cortisol	<10	<10	16 ± 1	2690 ± 288
11-Deoxycortisol	<10	<10	<10	122 ± 39
Corticosterone	<10	<10	175 ± 16	174 ± 63
Dehydroepiandrosterone	<10	<10	<10	83.6 ± 2.6
Androstenedione	<10	<10	<10	67.5 ± 6.3
Dehydroepiandrosterone sulphate	<50	<50	<50	1550 ± 154
Pregnenolone sulphate	<50	1440 ± 95	<50	1030 ± 182

Pregnenolone and progesterone were added as a single dose (20 μg) on the 12th day after changing the culture medium. ACTH was added daily at a dose of 0.02 IU/ml of culture medium for 6 days. Means ±S.E.M. of three cultures in each group are indicated. The units are ng/ml of tissue culture medium.

production. At the same time DHA increased (116%; $P = 0.023$) and androstenedione decreased (35.1%; $P = 0.097$). Figure 2 shows the effect of various concentrations of estriol on cortisol production. Estradiol-17β was also tested in lower concentrations (results not shown in figures). When estradiol-17β was added at a concentration of 0.2 μg/ml of tissue culture medium together with ACTH for six days, no significant reduction in cortisol production was noted. When estradiol was added as a single dose at a concentration of 4 μg/ml of tissue culture medium together with ACTH, there was a 54% reduction in cortisol production compared with cultures treated only with ACTH. Neither estriol-3-sulphate nor DHAS at a concentration of 20 μg/ml of tissue culture medium had any significant effect on steroidogenesis.

The effects of hCG and ACTH on adrenal steroidogenesis

Table 3 shows the effect of hCG on human fetal adrenal steroidogenesis during the first 6 days of cultivation. There was no stimulation of DHA or DHAS by hCG compared to the control cultures. Table 4 shows the effect of combined hCG + ACTH treatment compared to mere ACTH treatment. hCG did not change the steroid pattern towards DHA or DHAS production. The steroidogenic response to ACTH was clear also at this stage of cultivation.

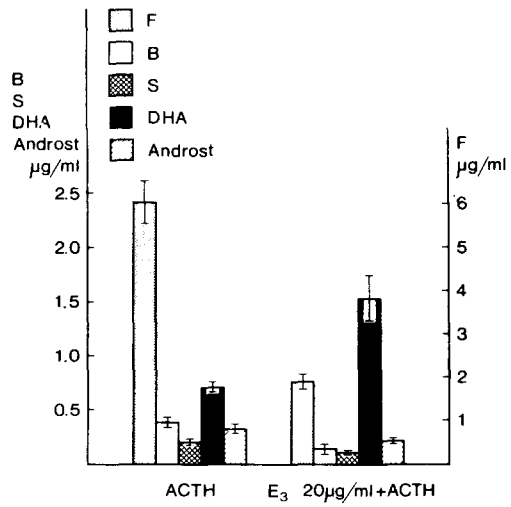

Fig. 1. The effects of estriol (E_3), (20 μg/ml of tissue culture medium) on ACTH-stimulated steroidogenesis in tissue culture of human fetal adrenals. Three cultures in each group were analysed, vertical lines indicate ±S.E.M. F = cortisol. B = corticosterone, S = 11-deoxycortisol. DHA = dehydroepiandrosterone, Androst = androstenedione.

DISCUSSION

The activity of 3β-HSD in the fetal zone of human adrenal cortex is very low. This is evident from histochemical experiments [e.g. 16, 17], although some authors have noted activity of this enzyme [18]. The secretory pattern of human fetal adrenals is also well known [for reviews see e.g. 1–3] and both incubation [4] and tissue culture [6] experiments show the lack of 3β-HSD. In the present study addition of pregnenolone into the tissue culture medium on the first day of cultivation did not increase cortisol production or other delta-4-keto-3 steroids significantly. Instead, an increase was noted in the production of sulphate conjugates of pregnenolone and DHA. Thus the lack of activity of 3β-OH-steroid dehydrogenase

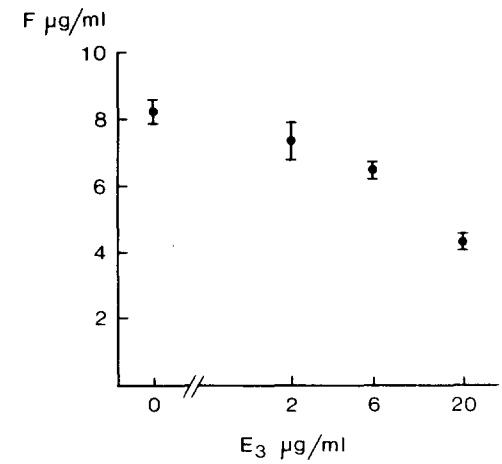

Fig. 2. The effect of various concentrations of estriol (E_3) on cortisol (F) production in ACTH-stimulated (0.02 IU/ml/day for 6 days) tissue culture of human fetal adrenals. Three cultures in each group were analysed, vertical lines indicate ±S.E.M.

Table 3. The effect of hCG (1 IU/ml/day) on endogenous steroid production during the first six days of cultivation of human fetal adrenals

	Control (three cultures)	hCG (three cultures)
Cortisol	35.7*	30.5*
Dehydroepiandrosterone (DHA)	36.1*	29.6*
Androstenedione	21.0*	22.0*
Dehydroepiandrosterone sulphate (DHAS)	2710 ± 302	2900 ± 340
Pregnenolone sulphate	679 ± 112	665 ± 112

* Analysed from pooled extracts of three cultures.

The values are ng/ml of tissue culture medium and means ±S.E.M. in each group are indicated.

Table 4. The effect of combined hCG + ACTH treatment compared to only ACTH treatment (and to controls without any treatment) in tissue culture of human fetal adrenals during the first six days of cultivation (hCG 1 IU/ml/day. ACTH 0.02 IU/ml/day)

	ACTH (three cultures)	hCG + ACTH (three cultures)	Control (three cultures)
Cortisol	1930 ± 76	1620 ± 227	94.3 ± 7.0
Corticosterone	62.8 ± 8.8	53.4 ± 4.2	< 10
11-Deoxycortisol	178 ± 30	216 ± 37	< 10
Dehydroepiandrosterone (DHA)	171 ± 25	115 ± 20	< 10
Androstenedione	130 ± 19	145 ± 33	< 10
Dehydroepiandrosterone sulphate (DHAS)	3370 ± 305	2740 ± 117	2200 ± 84
Pregnenolone sulphate	1330 ± 225	1140 ± 155	467 ± 25

The values are ng/ml of tissue culture medium and means ±S.E.M. in each group are indicated.

delta-5-delta-4 isomerase is maintained in tissue culture of human fetal adrenals.

When progesterone was added to the culture medium on the first day of cultivation, a major part of this was converted into several delta-4-keto-3 steroids during the first days of cultivation. Cortisol was quantitatively the most important product. It can be calculated that more than half of the added progesterone was converted into the delta-4-keto-3 steroids analysed here (cortisol, corticosterone, 11-deoxycortisol and androstenedione). During later stages of cultivation, when the fetal zone cells are no longer visible in the culture and when the proliferating adrenal cells represent the morphologically undifferentiated permanent cortex type [5], conversion of progesterone into cortisol was very limited. The lower conversion of progesterone into cortisol than corticosterone shows the low activity of 17α-hydroxylase at this stage of cultivation; however ACTH also activates this enzyme. The sulphokinase system also seems to be working in these ultrastructurally undifferentiated permanent cortex cells, because pregnenolone sulphate was formed when pregnenolone was added to the culture medium at this stage of cultivation. DHAS, however, was not formed. On the basis of the present study the fetal human adrenal, and probably especially the fetal zone, has in addition to being able to form large amounts of sulphate conjugates of delta-5 steroids, a high capacity to convert exogenous progesterone into cortisol and other corticosteroids. Our results are in accordance with those of Villee and Driscoll[19] and Whitehouse and Vinson[20], who studied the conversion of [7α-^{3}H]-pregnenolone and [4-^{14}C]-progesterone in incubations of minced human fetal adrenals. In perfusion studies of previable human fetuses it has been shown that when [4-^{14}C]-progesterone was infused into mid-gestation human fetuses, the uptake of radioactivity was very high in the adrenal cortex [21]. It has also been shown that progesterone concentration in venous cord blood is much higher than in arterial cord blood [22, 23]. Thus the human fetal adrenal is capable of synthesizing cortisol in spite of inefficient 3β-HSD using placental progesterone as a substrate. The quantitative significance of this cortisol to the fetus as regards placental transfer of maternal cortisol cannot be estimated on the basis of the present study. Our results also show the capability of human fetal adrenals to form estrogen precursors (DHAS) for placental aromatisation from pregnenolone.

The factor(s) responsible for the lack of enzymatic activity of 3β-HSD remains an enigma. Villee[24, 25] has suggested that progesterone may be responsible for depression of this enzyme. Goldman[7] gave large doses of estradiol-17β to pregnant rats. This caused congenital adrenal hyperplasia in fetuses, and this was shown to be due to inhibition of 3β-HSD. The present study shows that estrogens (estriol, estradiol-17β) inhibit secretion of steroids of the delta-4-keto-3 series and simultaneously increase secretion of the delta-5 steroids (e.g. DHA). It is therefore evident that the site of action of estrogens is 3β-HSD. When the possible physiologic role of estrogens as depressors of 3β-HSD in human fetal adrenals *in vivo* is considered,

the inhibitory concentration used in the present study must be discussed. The inhibitory concentration of estriol in the present study in ACTH-stimulated tissue culture was of the order of 2–20 μg/ml (= 0.7–7.0 × 10^{-5} M). Estriol concentration in venous cord blood is greater than in arterial cord blood and it is about 140 ng/ml (= 4.9 × 10^{-7} M)[23]. The estradiol concentration used was also unphysiologic. Thus the inhibitory concentrations of estrogens found in the present study are not physiologic *in vivo*. On the other hand, the concentration of ACTH used was also supraphysiologic. If estrogens are supposed to be responsible for the depression of 3β-HSD in human fetal adrenal *in vivo*, the sensitivity of the fetal zone cells *in vivo* during fetal development must be much higher than that of the cultured fetal adrenal cells (mainly of permanent cortex type) in the present study. The depression (or lack) of 3β-HSD in fetal zone cells is irreversible, as shown by the fact that this enzyme does not become activated when fetal adrenals are cultured *in vitro*. The inhibition of 3β-HSD by estrogens in our tissue culture experiment is probably qualitatively different from that of the fetal zone cells *in vivo*, because our inhibition is not so absolute, demands unphysiologic concentrations of estrogens and is reversible (if estrogens are removed from the tissue culture medium, the inhibition disappears). Our results, however, show that estrogens are able to inhibit steroidogenesis and that the step of inhibition is preferentially 3β-OH-steroid dehydrogenase delta-5-delta-4 isomerase.

Some authors have suggested that hCG is the physiologic stimulus for the fetal zone of human adrenal [8, 9, 26]. This is a tempting thought for several reasons: The concentration of hCG in the fetal organism is highest during the first trimester of gestation [27] when the fetal adrenals begin to function. There is some evidence that in anencephalics the fetal zone usually develops normally up to the fifth month of pregnancy in spite of the obvious absence of ACTH secretion [28]. The actual experimental evidence for the stimulatory effect of hCG on DHA or DHAS production is scanty. Johannisson[26] gave very large doses of hCG intraamniotically to human fetuses and found morphological changes in fetal adrenals which indicated increased secretory activity. Administration of hCG to newborns increased the excretion of DHA in urine [29]. Lehman and Lauritzen[8] have shown increased conversion of pregnenolone and cholesterol to DHA in incubated slices of human fetal adrenals when hCG was present. Jaffe *et al.*[9] have reported increased DHAS production from fetal zone using superfusion system under influence of hCG. The methodology for DHAS measurement was not, however, specific, but also measured other androgens. In the present study we could not show any increase of DHA or DHAS in the hCG-treated cultures compared to the controls. Neither did hCG modify the stimulatory effect of ACTH towards increased DHA or DHAS production. Our studies concerned mid-term fetal adrenals and so the possibility remains that hCG may have some effect on fetal adrenals during the first trimester of pregnancy. hCG is not without effect on fetal endocrinology, because it stimulates testosterone production from fetal testis [30–32]. ACTH showed a very clear stimulatory effect on both free and sulphate-conjugated steroid production at all stages of cultivation indicating the activation of all necessary enzymes for adrenal steroid synthesis (including 3β-HSD). We propose that the stimulus for human fetal adrenals is ACTH and that some (maybe placental) factor(s) other than hCG in a one way or another blocks 3β-OH-steroid dehydrogenase in the fetal zone.

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