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ONTOGENESIS OF CHOLESTEROL SIDE-CHAIN CLEAVAGE ACTIVITY IN THE OVINE ADRENAL DURING LATE GESTATION

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Summary—The present study examined the activity of the cholesterol side-chain cleavage system, and the amount of cytochrome *P*450scc in adrenal glands of sheep fetuses and newborn lambs as well as the *in vitro* regulation of these parameters. Freshly isolated fetal adrenal cells incubated in the presence of 1 mM 8Br-cAMP or 25 μM 22R-OH cholesterol, produced 4- to 5-fold less pregnenolone than neonatal cells under similar conditions. Likewise, pregnenolone production by isolated fetal adrenal mitochondria was lower than that of neonatal mitochondria when endogenous cholesterol was used as a substrate or when 22R-OH cholesterol was added to the incubation medium. Also, the amount of *P*450scc, determined by immunoblot, was lower in fetal mitochondria than in neonatal mitochondria. In culture, ACTH, despite enhancing both the production of pregnenolone and the incorporation of [^{14}C]acetate in cholesterol and its end-products by fetal adrenal cells, neither increased the amount of pregnenolone formed from 22R-OH cholesterol nor the amount of immunoreactive *P*450scc. By contrast, during the first 48 h of culture under standard conditions, there was a "spontaneous" increase in the activity of *P*450scc which reached values observed in neonatal adrenal cells. Such a development was inhibited when 5% ovine fetal serum was added to the culture medium. These results reinforce the view that in the ovine fetal adrenal gland, the development of *P*450scc is not ACTH-dependent but involves most probably a decrease in inhibitory factors present in fetal blood.

INTRODUCTION

In mammalian species, the fetal adrenal gland plays a key role during the last third of gestation since glucocorticoids are involved in the maturation of many fetal organ systems [1]. Moreover, in the sheep and in other domestic ruminants as well as in the pig, the onset of parturition depends on an increase in the level of corticosteroids in the fetal blood [2, 3]. In the sheep, this increase occurs from about 130 days onwards and results from an enhanced activity of the fetal adrenal cortex [4, 5] which in turn is due to both an increase in tropic stimulation of the gland [6, 7] and to a dramatic development of the steroidogenic response to ACTH of fetal adrenocortical cells [8]. The increasing capability of fetal adrenal cells to produce corticosteroids before birth in this species, appears mainly related to alterations occurring early in the steroidogenic pathway which lead to an increased production of pregnenolone [9, 10]. Such a phenomenon could be due to 2 processes: (i) increased amounts of cholesterol available for steroidogenesis; or (ii) enhanced conversion of cholesterol to pregnenolone. In previous studies, we observed that adrenocortical cells from 115- to 127-day-old ovine fetuses or from newborn lambs could use cholesterol from both low and high density lipoproteins for steroidogenesis and that, *in vivo*, cholesterol availability in the blood was not a limiting factor for adrenal steroidogenesis of these animals [11]. In the present work therefore, we have studied the activity of the cholesterol side-chain cleavage (CSCC) system and the relative amount of cytochrome *P*450scc in the adrenal glands of sheep fetuses at the end of gestation. Some experiments on the regulation of this activity are also reported.

MATERIALS AND METHODS

Animals

Crossbred (Ile de France × Romanov-Ile de France) fetuses (89- to 144-day-old) and lambs (3- to 10-day-old) were used throughout. The

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normal gestation period of this breed is 145 ± 1 days. Fetuses were obtained by caesarean section under general anesthesia of the mother (fluothane/O_2). Animals were killed by decapitation and pairs of adrenals were quickly removed and kept in physiological saline at 0°C until processed. In each experiment 10 to 40 adrenal glands were pooled.

Adrenal cell isolation, culture and incubation

Adrenal cells were isolated and cultured in 1 cm^2 multiwell culture plates (Nunclon Delta, Polylabo, Strasbourg, France) at a starting density of 3×10^5 cells/well as described previously [9, 12]. Media were renewed daily and $ACTH_{1-24}$ (10^{-8} M) (Synacthene, Ciba, Rueil-Malmaison, France) or fetal ovine serum were added to the appropriate wells from day 1 onwards.

For Western blot analysis, cells were cultured as above but in 100 mm Petri dishes (Nunclon Delta) at a starting density of 10^7 cells/dish.

At selected times during the culture, cells were incubated for 2 h at 37°C in the presence of the enzymatic inhibitors, WIN 24540 (4α,5-epoxy-17β-hydroxy-5α-androstane; Winthrop Labs, France), 4 μM, a blocker of the 3β-hydroxysteroid dehydrogenase isomerase activity and SU 10603 [7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(24)-naphthalen-one, Ciba-Geigy corporation, Summit, NJ, U.S.A.], 5 μM, a blocker of the 17α-hydroxylase activity, and with or without 1 mM 8Br-cAMP (Sigma chimie Sarl, La Verpillière, France) or 25 μM 22R-OH cholesterol (Sigma). At the end of the incubation period, the medium was withdrawn and stored at −20°C until assayed for pregnenolone content. At the end of the experiments, cells were detached in NaCl 0.9%, 1 mM EDTA pH 7.4, 0.1% trypsin (Worthington, Eurobio, Les Ulis, France) and counted in a coulter counter (Coultronics France SA., Margency).

When freshly isolated cells were used for incubation (day 0), cells were preincubated for 90 min at 37°C and then incubated for 2 h in the absence or presence of 22R-OH cholesterol, or ACTH or 8Br-cAMP, under gentle shaking [12].

Incorporation of [^{14}C]acetate into cholesterol pools and assay of HMG-CoA reductase activity

These were performed as described previously [13].

Preparation of subcellular fractions

Cell homogenates. Cells were washed once and then scraped in physiological saline at room temperature. After a 5 min centrifugation at 100 *g*, the pellets were resuspended in a small volume of hypotonic buffer, Tris–HCl 10 mM, KCl 10 mM, EDTA 0.5 mM, pH 7.4 (about 200 μl for 5×10^6 cells) and sonicated (10 μ peak to peak, for 2×10 s). The cell homogenates were then assayed for protein content.

Mitochondrial fractions. These were prepared from adrenal cortices according to Lambert *et al.* [14], in a Tris–HCl 20 mM, sucrose 250 mM, pH 7.4 buffer. Mitochondrial pellets were resuspended in a small volume of the same buffer and were assayed for protein content. These mitochondrial fractions were either used for pregnenolone production when incubated according to Lambert *et al.* [14] or for *P*450scc immunoreactive quantitation by Western blotting.

Western blot analysis

Proteins in cell or mitochondrial homogenates were separated by SDS–PAGE as described by Laemmli [15] using a Bio-Rad apparatus. 5 μg of proteins were loaded in each lane of a 12% gel and migration lasted 4 h at 140 V. Separated proteins were transferred to nitrocellulose (Bioblock Scientific, Illkirch, France) at 0.8 mA/cm^2 of gel, overnight in Tris–HCl 25 mM, glycine 192 mM, pH 8.3, 0.01% SDS and 20% methanol as described by Towbin *et al.* [16], using a mini Transphor TE 22 from Hoeffer (Bioblock, France).

The remaining nonspecific binding sites on nitrocellulose were blocked with T1 buffer [Tris–HCl 10 mM, pH 8.8, 1% bovine serum albumin (BSA), 0.3% Tween 20, 1% goat serum] at room temperature for at least 1 h. The sheet was then incubated with 50 ml T1 buffer containing anti-*P*450scc (kind gift from Dr D. B. Hales, Ann Arbor, MI, U.S.A.) [17] diluted 1/10,000 for 1 h at room temperature. Unbound antibody was removed by washing 3 times with the same buffer devoid of Tween 20 (T2). The sheet was then incubated with a secondary anti-rabbit IgG (Institut Pasteur, Marnes-la-coquette, France), labeled with peroxidase, diluted 1/1000 in T2, for 1 h at room temperature. The blot was then washed 3 times with Tris–HCl 10 mM, pH 8.8 and incubated in a freshly prepared solution of 10 mg 3,3′ diaminobenzidine (Sigma) in 50 ml Tris–HCl buffer and 50 μl H_2O_2 (30% w/v). The reaction was terminated by washing the blot in tap water.

Blots were scanned with a scanning densitometer (Schimadzu, Japan).

Protein assay

The protein content was assayed following the Bradford procedure [18]. BSA (Sigma) was used as a reference standard.

Radioimmunoassay of pregnenolone

The collected media were boiled for 10 min to denature binding proteins. Pregnenolone RIA was performed according to De Peretti and Mappus [9] who kindly provided the antibody.

Analysis of results

Student's paired t-test was used to compare pregnenolone outputs by fetal adrenal isolated cells incubated with or without 22R-OH cholesterol or 8Br-cAMP, or cultured in the absence or presence of ACTH or of ovine fetal serum. When required, pregnenolone productions were logarithmically transformed to eliminate heterogeneity of variances. Analysis of variance was used to test for significance of differences in other cases. In each case, n corresponded to the number of experiments.

RESULTS

CSCC activity in freshly isolated material

Pregnenolone productions by freshly isolated fetal and neonatal adrenal cells (day 0) incubated in the absence or presence of either 8Br-cAMP or 22R-OH cholesterol, are given in Table 1. Basal production was low for both fetal and neonatal adrenal cells and was increased in the presence of 8Br-cAMP for neonatal cells only ($P < 0.003$). In order to determine whether the amount of available cholesterol could be of importance in regard to this fetal lower production, cells were incubated in the presence of 22R-OH cholesterol in excess. In this case, pregnenolone production was increased 18-fold for fetal cells ($P < 0.005$) and 16-fold for neonatal cells ($P < 0.03$) as compared to 8Br-cAMP-stimulated pregnenolone production. However, pregnenolone production by fetal cells remained lower than that of newborn lambs ($P < 0.03$).

Table 1. Pregnenolone production (expressed in ng/2 × 10^5 cells/2 h incubation) by freshly isolated ovine fetal and neonatal adrenal cells

	Fetus	Newborn
Basal	3.2 ± 0.8	4.7 ± 0.8
22R-OH cholesterol	90 ± 13[b]	368 ± 57[c]
8Br-cAMP	4.9 ± 1.0	23 ± 0.3[a]

Adrenal cells from ovine fetuses or newborn lambs were isolated, then incubated for 2 h in the absence (basal) or presence of either 25 μM 22R-OH cholesterol or 1 mM 8Br-cAMP and in the presence of inhibitors of pregnenolone metabolism (see Materials and Methods). At the end of the incubation period, media were withdrawn and pregnenolone contents were determined. Values represent means ± SEM of 4 experiments for fetuses and 3 experiments for newborn lambs. Each condition was tested in triplicate.

[a] $P < 0.003$ vs corresponding basal value.

[b] $P < 0.005$ vs corresponding 8Br-cAMP-stimulated value.

[c] $P < 0.03$ vs corresponding 8Br-cAMP-stimulated value and vs corresponding fetal value.

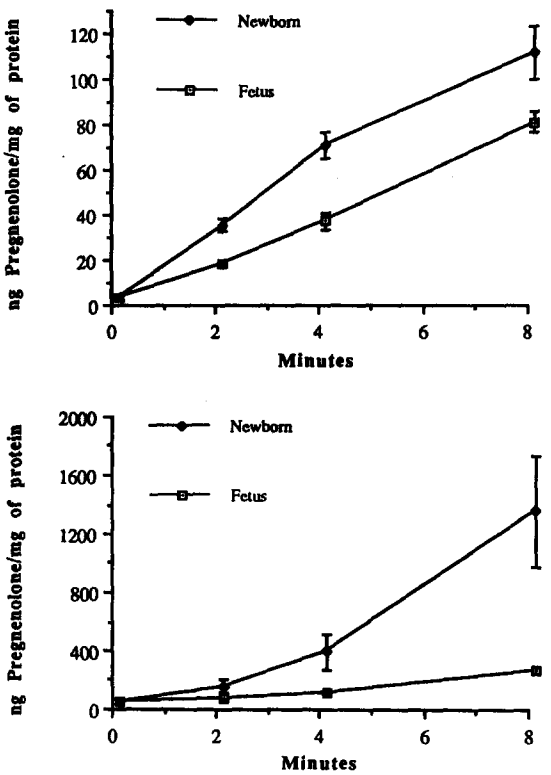

Fig. 1. Pregnenolone production by isolated ovine fetal and neonatal adrenal mitochondria, incubated in the absence (top) or presence (bottom) of 22R-OH cholesterol. Each point is the mean ± SEM of 4 experiments each performed in triplicate. In the absence of 22R-OH cholesterol, differences between fetuses and newborn lambs were significative ($P < 0.002$ at 2 min, $P < 0.03$ at 4 min and $P < 0.05$ at 8 min). In the presence of 22R-OH cholesterol, differences were significant at $P < 0.06$ (4 min) and $P < 0.03$ (8 min).

In order to be sure that the lower metabolization of 22R-OH cholesterol by fetal cells was specific of the CSCC activity, we next studied the production of pregnenolone by fetal and neonatal mitochondria incubated *in vitro*. For this purpose, mitochondria were isolated from fetal and neonatal adrenal cortices and incubated in the absence or presence of an excess of 22R-OH cholesterol. The results, presented in Fig. 1, show that pregnenolone production by fetal adrenal mitochondria was 2-fold lower than that of neonatal mitochondria when endogenous cholesterol was used. In the presence of 22R-OH cholesterol, the amount of pregnenolone produced by fetal mitochondria

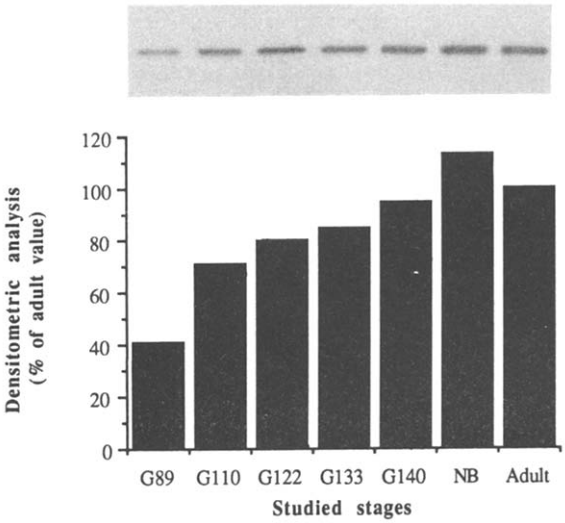

Fig. 2. Comparative quantification of immunoreactive *P*450scc present in adrenal mitochondria from ovine fetuses of various ages (89 to 140 days of gestation), from newborn lambs (NB) and from adult sheep. Densitometric values were calculated relatively to the value obtained with the adult which was considered as equal to 1. 2 to 20 adrenal glands were pooled for mitochondrial preparations and the Western blot was performed 3 times.

was about 5-fold lower than that of mitochondria from newborn lambs (at 8 min of incubation). Hence, in this system, cholesterol availability seemed to be more limiting for neonatal than for fetal pregnenolone production.

We next tried to find out if the fetal lower CSCC activity could be due to a lower amount of *P*450scc present in fetal mitochondria. Therefore, immunoreactive *P*450scc present in adrenal cortex mitochondria from fetuses of various ages, from newborn lambs and from adult sheep was assessed by immunoblotting (Fig. 2). The relative amount of *P*450scc increased almost 3-fold between 89 days of gestation and birth, and about 1.5-fold between 122 days of gestation and birth (similar results were obtained with another antibody, gift of Dr E Chambaz, Grenoble, France; data not shown).

In an attempt to better understand the regulation of pregnenolone production by the ovine fetal adrenal gland, we next studied the effect of ACTH treatment on both pregnenolone production and on the amount of cytochrome *P*450scc present in those fetal adrenal cells in culture.

In vitro regulation of fetal adrenal pregnenolone production by ACTH

On day 2 of culture, pregnenolone production by control ovine fetal adrenal cells stimulated for 2 h by 8Br-cAMP, was about 6-fold higher than the basal production (Table 2) but this increase was not significant. By contrast, on day 6, 8Br-cAMP-stimulated pregnenolone production was significantly increased ($P < 0.04$) over basal value even though the basal output had increased 8-fold between days 2 and 6 ($P < 0.06$). The presence of ACTH in the culture medium for 24 h between days 1 and 2 allowed a 2- to 4-fold increase of the production of pregnenolone stimulated by 8Br-cAMP when compared to that of cells cultured in the absence of ACTH ($P < 0.01$). A 5 day-ACTH-treatment allowed the 8Br-cAMP-stimulated pregnenolone production to double on day 6 of culture when compared to that of cells cultured in the absence of ACTH ($P < 0.02$), and pregnenolone concentration under these conditions became 4-fold more important than on day 2 ($P < 0.01$). When cells were incubated in the presence of 22R-OH cholesterol, the amount of pregnenolone produced by cells cultured in the presence of ACTH was never higher than that of control cells and no increase of pregnenolone production was observed between days 2 and 6. In order to make sure that this lack of ACTH effect was not due to an inappropriate length of treatment by the hormone (desensitization?), various times of culture in the presence of this hormone were tested. ACTH was never able to increase pregnenolone production by cells incubated in the presence of an excess of 22R-OH cholesterol with treatment periods of 24 to 96 h (data not shown).

Table 2. Pregnenolone production by ovine fetal adrenal cells (expressed in ng/2 × 10^5 cells/2 h incubation) on the 2nd (day 2) and 6th day (day 6) of culture

Treatment	Incubation	Pregnenolone
	Day 2	
Control	Basal	1.1 ± 0.1
Control	22R-OH cholesterol	533 ± 105
ACTH 10^{-8} M	22R-OH cholesterol	525 ± 145
Control	8Br-cAMP	7.5 ± 5.9
ACTH 10^{-8} M	8Br-cAMP	28.3 ± 18.5[b]
	Day 6	
Control	Basal	8.7 ± 3.8
Control	22R-OH cholesterol	288 ± 159
ACTH 10^{-8} M	22R-OH cholesterol	342 ± 89
Control	8Br-cAMP	50.5 ± 9.8[a]
ACTH 10^{-8} M	8Br-cAMP	119.5 ± 5.3[b]

Cells were cultured for 2 or 6 days in the absence (control) or presence of ACTH. On day 2 or 6 of the experiment, cells were incubated for 2 h in the absence (basal) or presence of either 25 μM 22R-OH cholesterol or 1 mM 8Br-cAMP, and their production of pregnenolone was determined. Values are the mean ± SEM of 4 experiments on day 2 and 3 experiments on day 6. Each treatment was performed in 3 dishes which were assayed each in triplicate.

[a] $P < 0.04$ vs corresponding value for control cells.

[b] $P < 0.04$ vs corresponding basal value.

Table 3. Effect of an *in vitro* treatment with ACTH, on cholesterol metabolization (expressed in ^{14}C cpm incorporated/2 × 10^5 cells) and on HMG-CoA reductase activity (expressed as nmol/2 × 10^5 cells/20 min) of ovine fetal adrenal cells cultured for 2 or 6 days

Conditions	EC	FC	P5	P4	6 steroids	Total cpm	HMGCoA red. Act
D2 control	1960 ± 507	21350 ± 2021	371 ± 51	3332 ± 333	12245 ± 1944	39258	1.25 ± 0.26
D2 ACTH-treated	3653 ± 1242	18181 ± 1770	613 ± 123[a]	4024 ± 393	18192 ± 3070[a]	44663	1.21 ± 0.23
D6 control	994 ± 228	25723 ± 3540	229 ± 44	3218 ± 404	12792 ± 1229	42876	0.43 ± 0.05
D6 ACTH-treated	9730 ± 1681[a]	62548 ± 6771[a]	1851 ± 552[a]	24786 ± 6602[a]	28347 ± 2575[a]	127262	0.71 ± 0.06[a]

Cells were cultured for 2 (D2) or 6 (D6) days in the absence (control) or presence of ACTH (10^{-8} M), then incubated for 2 h in the presence of ^{14}C-labeled acetate and harvested. ^{14}C-labeled metabolites and HMG-CoA reductase activity were determined as described in Materials and Methods. Values for all compounds [esterified cholesterol (EC), free cholesterol (FC), pregnenolone (P5), progesterone (P4) and 6 steroids (11-deoxycorticosterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone, cortisol, 11-deoxycortisol and corticosterone)] are the mean incorporated cpm ± SEM of 4 experiments, each treatment being tested in triplicate. HMG-CoA reductase values are the mean ± SEM of 2 experiments, each performed in triplicate.

[a] $P < 0.02$ to $P < 0.004$ vs corresponding control value.

Pregnenolone production by control fetal adrenal cells incubated in the presence of 22R-OH cholesterol on day 2 was 6 times more important than pregnenolone production on day 0 ($P < 0.006$) (compare to Table 1).

Moreover, ACTH was not able to increase the amount of immunoreactive *P*450scc present in cultured adrenal cells, irrespective of the length of treatment by this hormone (data not shown).

Since the above results suggested an effect of ACTH treatment at the cholesterol level, the effect of such a treatment was studied on 2 parameters: (i) [^{14}C]acetate incorporation in cholesterol and its end-products, and (ii) HMG-CoA reductase activity. Results are given in Table 3. A significant difference was not observed between control and ACTH-treated cells on day 2 of culture for all parameters. However, on day 6, [^{14}C]acetate incorporation in all studied steroids was increased significantly ($P < 0.02$ to $P < 0.004$) in ACTH-treated cells and HMG-CoA reductase activity was doubled ($P < 0.004$) when compared to that of control cells.

Development of CSCC activity during the first 2 days of culture

Pregnenolone productions by fetal and neonatal adrenal cells, cultured under control conditions and either stimulated for 2 h by 8Br-cAMP or incubated with 22R-OH cholesterol, on days 0, 1 and 2 of culture, are given in Table 4. When fetal cells were stimulated by 8Br-cAMP, no difference was observed in term of pregnenolone production between days 0, 1 and 2 of culture. The same way, the response of neonatal cells on day 2 was not significantly different from the one obtained on day 0, but it was 2- to 4-fold higher than the response of fetal cells on day 0 ($P < 0.001$). By contrast, when adrenal cells were incubated in the presence of 22R-OH cholesterol, fetal cells produced 6 times more pregnenolone on day 2 ($P < 0.04$) than on day 0. On the opposite, neonatal cells produced the same amount of pregnenolone every day.

Despite the fact that, on day 0, fetal cells incubated with either 8Br-cAMP or 22R-OH cholesterol produced 5-fold less pregnenolone than neonatal cells, from day 1 on, fetal cells produced as much pregnenolone as neonatal cells, no matter what the incubation conditions were.

Inhibitory effect of fetal ovine serum on the development of CSCC activity in ovine fetal adrenal cells in vitro

In order to determine why development of fetal CSCC activity occurred during the first 2 days of culture, fetal adrenal cells were plated in medium containing 2% fetal ovine serum (FOS), then cultured from days 1 to 2 in medium supplemented with 5% either FOS (from 111-day-old fetuses) or adult ovine serum (AOS). On day 2, pregnenolone production by fetal adrenal cells cultured in the presence of AOS and incubated with 22R-OH cholesterol tended to be higher than pregnenolone production on day 1 ($P < 0.07$) (Table 5). When the

Table 4. Pregnenolone production (expressed in ng/2 × 10^5 cells/2 h incubation) by ovine fetal and neonatal adrenal cells on days 0, 1 and 2 of culture

	Day 0	Day 1	Day 2
Fetus			
Basal	3.2 ± 0.8	1.95 ± 0.9	1.1 ± 0.1
22R-OH cholesterol	90 ± 13	434 ± 131[b]	533 ± 105
8Br-cAMP	4.9 ± 1.0	4.0 ± 2.2	7.5 ± 5.9
Newborn			
Basal	4.7 ± 0.8	0.8 ± 0.2	1.6 ± 0.3
22R-OH cholesterol	368 ± 57[a]	346 ± 208	372 ± 45
8Br-cAMP	23 ± 0.3[c]	6.4 ± 0.8	11.8 ± 7.4

Adrenal cells from ovine fetuses or newborn lambs, freshly isolated (day 0) or cultured under control conditions for 1 (day 1) or 2 (day 2) days were incubated for 2 h in the absence (basal) or presence of either 22R-OH cholesterol or 8Br-cAMP and their production of pregnenolone was assessed. Values are the mean ± SEM of 4 experiments for fetuses and of 3 experiments for newborn lambs, each condition being performed in 3 dishes and each dish being assayed in triplicate.

[a] $P < 0.001$ vs corresponding value for fetal cells.

[b] $P < 0.04$ vs corresponding fetal value on day 0.

[c] $P < 0.003$ vs corresponding fetal value.

Table 5. Effect of the presence of FOS or AOS in the culture medium on the development of CSCC activity of ovine fetal adrenal cells (pregnenolone production is expressed in ng/200,000 cells/2 h incubation)

Day of culture	Conditions	Incubation	Pregnenolone
1	FOS 2%	Basal	0.6 ± 0.11
		22R-OH cholesterol	287 ± 75[a]
2	AOS 5%	Basal	2.7 ± 1.1
		22R-OH cholesterol	711 ± 174
2	FOS 5%	Basal	3.9 ± 1.7
		22R-OH cholesterol	433 ± 151[b]

Fetal adrenal cells were plated in medium containing 2% FOS, then cultured between days 1 and 2 in the presence of either 5% AOS or FOS. On days 1 and 2, cells were incubated for 2 h in the absence (basal) or presence of 25 μM 22R-OH cholesterol and their production of pregnenolone was assessed. Values are the mean ± SEM of 3 experiments on day 1 and of 4 experiments on day 2, each performed in triplicate.

[a] $P < 0.07$ vs corresponding value on day 2 in the presence of AOS.

[b] $P < 0.002$ vs corresponding value on day 2 in the presence of AOS and not significative vs corresponding value on day 1.

culture was performed in the presence of FOS, no enhancement of pregnenolone production was observed between days 1 and 2, but the value obtained on day 2 was significantly lower than when the culture was performed with AOS ($P < 0.002$). Similar results were obtained with FOS from either 98- or 119-day-old fetuses (data not shown).

DISCUSSION

Previous studies [9, 10] have shown indirectly that the development of the steroidogenic capacities of ovine fetal adrenal cells during the last month of gestation are correlated to an increased ability of these cells to produce pregnenolone. Moreover, the regulation of the capacity of these cells to produce pregnenolone was also shown to be only partially dependent on ACTH [20]. Our results confirm directly by assaying pregnenolone production, the above previous observations and point out some mechanisms involved in this development. Besides, it must be noted that the blocker of the 17α-hydroxylase activity used throughout the present experiments (SU 10603) is much more specific of this enzymatic activity [21] than was spironolactone which was previously used [20].

Several factors could be responsible for the lower pregnenolone production by 8Br-cAMP-stimulated freshly isolated fetal adrenal cells when compared to that of neonatal cells: (i) a less efficient protein kinase A (PKA) system in fetuses than in newborn lambs [22], (ii) a lower pool of cholesterol available for steroidogenesis in fetuses or (iii) a more limiting CSCC activity in fetal mitochondria due to either *P*450scc itself or to its associated enzymes.

Since fetal adrenal cells incubated with either 8Br-cAMP or 22R-OH cholesterol produced 4-fold less pregnenolone than neonatal cells, the first 2 propositions are unlikely and this matches former studies [11, 13, 21]. However, this result taken with those obtained with isolated mitochondria indicate that the CSCC system is indeed less efficient in fetuses than in newborn lambs. Moreover, considering the results of immunoblots, this deficient CSCC activity in fetuses might be due, at least partly, to a lower amount of cytochrome *P*450scc present in fetal than in neonatal mitochondria. This last point is not in agreement with the results of John *et al.* [23] who mentioned identical amounts of *P*450scc in total adrenal homogenates of ovine fetuses at 85, 127 and 145 days of gestation. But as a matter of fact, we used mitochondria preparations issued from adrenal cortex (instead of whole adrenal homogenates) which could contribute to explaining such a discrepancy.

It is however difficult to simply correlate the relatively slight increase of immunoreactive *P*450scc (reaching a maximum of 3-fold between 89 days of gestation and birth) presented in this paper, with a much more important increase of *P*450scc encoding mRNAs, which was observed by Tangalakis *et al.* [24] during the same period. This difference could indicate a "delay" in, or a "blocking" of, mRNA translation into protein. So the increase in mRNA would not be directly correlated to an increased production of the corresponding protein. Such a discrepancy has already been noted in the bovine adrenal cortex insofar as ACTH-induced mitochondria *P*450 enzymes are concerned [25]. In order to more precisely correlate CSCC activity through its ability to metabolize 22R-OH cholesterol and mitochondria amount of *P*450scc, it would be necessary to evaluate not only *P*450scc but also adrenodoxin and adrenodoxin reductase which are involved in the "CSCC system". This should also help to determine whether a post-translational "maturation or activation" of the CSCC itself is responsible for its increased activity in late gestation.

Concerning the enzyme regulation, we observed that CSCC activity was not altered by ACTH in the ovine fetal adrenal. This result is unexpected for several reasons. It has been clearly demonstrated, in the adult bovine adrenal, that ACTH increased:

(i) CSCC gene transcription [26],

(ii) the stability of *P*450scc-encoding mRNAs [27] and

(iii) the amount and activity of the enzyme [28]

In the human fetal adrenal also, ACTH has been shown to increase most of these parameters [26, 29, 30]. Moreover, in the ovine fetus, an ACTH infusion *in vivo* strikingly increases both the amount of *P*450scc-encoding mRNAs [31] and the adrenal cells ability to produce pregnenolone [9].

In our experimental conditions, the presence of ACTH in the culture medium increased 8Br-cAMP-induced pregnenolone production in comparison with that of control cells. But no effect of ACTH was observed either on 22R-OH cholesterol metabolization or on *P*450scc immunoreactive amount, even if the culture was performed in the presence of serum and insulin (as in the present paper) or in the absence of one or both of these two compounds (data not shown). It therefore seems reasonable to conclude that the positive effect of ACTH observed when cells were stimulated by 8Br-cAMP, does not involve a development of CSCC itself but more likely an increase in cholesterol available for steroidogenesis. When cells were incubated in the presence of [^{14}C]acetate, a 5-day-ACTH-treatment *in vitro* was able to increase both the amount of radiolabeled cholesterol and other steroids and HMG-CoA reductase activity. Nevertheless, it should be noted that a 24 h-ACTH-treatment was not sufficient for such an increase, although 8Br-cAMP-stimulated pregnenolone production was increased 4-fold under these conditions.

Taken together all these results suggest that the increased *de novo* synthesis of cholesterol is not a trigger to this development. In the guinea-pig, Lambert *et al.* [14] have shown that the enhanced pregnenolone production by mitochondria from *in vivo* ACTH-treated animals involves, most probably, an increase in the synthesis of a proteic factor allowing cholesterol translocation to the CSCC system. By contrast, these authors could not demonstrate any ACTH effect on the CSCC activity itself.

Another important result of the present work is the enhancement of the metabolism of 22R-OH cholesterol by control fetal adrenal cells during the first 2 days of culture. This development cannot be due to insulin present in the culture medium (through its own receptors or those of IGFs) for at least two reasons: (i) a similar phenomenon was not observed with neonatal adrenal cells, and (ii) neither insulin nor IGF1 is able to increase CSCC activity *in vitro* in bovine adult adrenal [32] as in ovine fetal adrenal cells [33]. Hence, it seemed reasonable to retake the hypothesis proposed by Durand *et al.* [9] and Cathiard *et al.* [20] concerning the existence, in fetal blood, of extra-pituitary factors inhibiting adrenal pregnenolone synthesis. Such an hypothesis is substantiated by the preventing effect of FOS, added to the culture medium; on the development of CSCC activity during the first 2 days of culture. Experiments are in progress to identify the factors involved in this regulation.

In conclusion, our results reinforce the view of the existence of a multifactorial regulation of the development of the ability of fetal sheep adrenal cells to produce pregnenolone. The metabolization of endogenous cholesterol into pregnenolone needs ACTH trophic action to actively maintain the system of delivery of cholesterol to the CSCC system. By contrast, the enzymatic activity itself is not positively regulated by ACTH, but is inhibited by factor(s) present in the fetal blood.

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