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3β-HYDROXYSTEROID DEHYDROGENASE–ISOMERASE ACTIVITY IN BOVINE ADRENOCORTICAL CELLS IN CULTURE: LACK OF RESPONSE TO ACTH TREATMENT‡

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Summary—Primary cultures of bovine adrenocortical cells (BAC) were used to determine whether the adrenal microsomal 3β-hydroxysteroid dehydrogenase–isomerase complex (3β-HSD), like the 17α-hydroxylase (17-OHase), responded to ACTH treatment with an increase in activity. Both enzymes influence the steroidogenic path leading to 17α-hydroxyprogesterone formation and thus could affect adrenal androgen biosynthesis. 3β-HSD Activity in postmitochondrial supernatant fluid, homogenates or cell monolayers remained unchanged after cells had been maintained in 1 μM ACTH up to 48 h. Since ACTH exposure led to a marked increase in 17-OHase activity over the same time period, it is concluded that, under the conditions used, the 3β-HSD–isomerase complex in BAC is nonresponsive to tropic hormone treatment.

INTRODUCTION

The synthesis of corticosteroids by adrenocortical cells requires at least four distinct cytochrome P-450 steroid hydroxylase enzymes and the action of the 3β-hydroxysteroid dehydrogenase (3β-HSD) [EC 1.1.1.51] and the isomerase (3-oxoisomerase) enzyme [EC 5.3.3.1]. The 3β-HSD and isomerase enzymes are found as a complex in the adrenal microsomal fraction [1] along with the steroid hydroxylases, 17α-hydroxylase ($P\text{-}450_{17\alpha}$) and the C_{21}-hydroxylase ($P\text{-}450_{C21}$). The 3β-HSD and isomerase enzymes catalyze the two step conversion of Δ^5-3β-hydroxysteroids (as pregnenolone) to Δ^4-3-ketosteroids (as progesterone). In that conversion, the initial reaction, catalyzed by the pyridine nucleotide requiring 3β-HSD, is considered to be the rate limiting step. Adrenal mitochondria contain other steroid hydroxylase enzymes, the cholesterol side chain cleavage cytochrome P-450 ($P\text{-}450_{scc}$) and the 11-hydroxylase ($P\text{-}450_{11\beta}$). The 3β-HSD and isomerase activities also can be detected in adrenal mitochondria [1–3].

ACTH has been shown to exert both acute and chronic control over the steroidogenic capacity of the adrenal cortex, actions which involve the cytochrome P-450 enzymes [4–6]. The rapid or acute response to ACTH occurs within seconds or minutes producing an increased steroid secretion. This rapid response is brought about by an increased interaction between cholesterol and cytochrome $P\text{-}450_{scc}$ resulting in an acceleration of the rate limiting step, the conversion of cholesterol to pregnenolone [4, 7]. The long term or trophic effect of ACTH is to maintain optimal steroidogenic capacity of the adrenal gland. This trophic effect is seen in the ability of exogenous ACTH to prevent the decline in adrenal cytochrome P-450 levels and steroidogenic capacity which normally follows hypophysectomy [8]. Primary cultures of adrenocortical cells have served as useful models for the study of the trophic actions of ACTH. Addition of ACTH to cultures of bovine adrenocortical cells has been shown to induce the synthesis of steroidogenic enzymes in mitochondria ($P\text{-}450_{scc}$, $P\text{-}450_{11\beta}$, adrenodoxin reductase and adrenodoxin) and microsomes ($P\text{-}450_{17\alpha}$ and $P\text{-}450_{C21}$) [5, 6, 9, 11]. Although the action of ACTH increases the synthesis of both microsomal cytochromes $P\text{-}450_{17\alpha}$ and $P\text{-}450_{C21}$ only the activity of the 17α-hydroxylase (17-OHase) is markedly elevated [9–11]. The increase in 17-OHase accounts for the shift in corticosteroid secretion, from corticosterone to cortisol, observed when BAC are exposed to ACTH [12].

Both the 17-OHase and 3β-HSD are microsomal enzymes and can act on the same substrate, pregnenolone, forming 17α-hydroxypregnenolone or progesterone respectively. The regulation of the 3β-HSD–isomerase complex as well as the 17-OHase could determine the rate of formation of 17α-hydroxypregnenolone vs 17α-hydroxyproges-

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terone and thus influence adrenal androgen formation. We have used primary cultures of bovine adrenocortical cells to examine the possibility of differential regulation of these adrenal microsomal steroidogenic enzymes by ACTH. We find that in cultures or in homogenates or subcellular fractions prepared from primary cultures that the activity of the 3β-HSD–isomerase complex is unaffected by the presence of ACTH under conditions which elevate the 17α-hydroxylase activity.

EXPERIMENTAL

Preparation of primary cultures of bovine adrenocortical cells was carried out as previously described except for the absence of fibroblast growth factor [12]. Cells were plated onto 100 mm (or 35 mm) plastic dishes and cultured until confluence was attained, generally within 5–6 days. Each experiment was begun by adding 1 μM $ACTH_{1-24}$ (Cortrosyn, Organon Inc., West Orange, NJ) to half the number of dishes (ACTH-treated cells); the ACTH-free dishes serving as control cells. The medium in each dish was changed at 24 h intervals and cells were harvested at intervals between 24 and 48 h.

Prior to harvesting cells, the incubation medium in each dish was removed, and aliquots pooled and saved for the determination of cortisol by radioimmunoassay [9]. The cell layer in each dish was then washed twice with 5 ml aliquots of Gey's balanced salt solution. A 1 ml aliquot of homogenizing buffer, 0.25 M sucrose with 25 mM HEPES pH 7.4, was added to each dish and the cells scraped from the dish using a rubber policeman. Each suspension of cells was sedimented by centrifugation at 480 *g* for 6 min. The packed cells were transferred to a Potter–Elvehjem type tissue grinder with 3–6 ml homogenizing buffer. The cells were homogenized by 20 passes using a motor driven pestle. The homogenate was centrifuged at 480 *g* for 10 min to sediment unbroken cells and cell debris. That cell pellet was resuspended in homogenizing buffer and again homogenized after which the sedimentation at 480 *g* was repeated. The two supernatant fractions were combined and centrifuged at 19,000 *g* for 15 min to sediment the mitochondrial fraction. The supernatant fluid from 19,000 *g* centrifugation was termed the postmitochondrial supernatant fraction (PMS). Protein content of homogenates, subcellular fractions, or cells removed from dishes was determined using the method of Bradford[13].

Enzyme assays

The assay procedure for the 17α-hydroxylase was carried out as previously described [9]. The assay of the 3β-HSD was carried out using a modification of the procedure of Schiebinger *et al.*[14]. The incubation mixture, total volume 1.0 ml, contained 0.5 mM NAD, with an NAD regenerating system of 5 mM pyruvate and 1 U lactate dehydrogenase, 50 nmol pregnenolone and 150–300 μg PMS protein; protein content was adjusted appropriately when the assay was performed using homogenate. The reaction was initiated by adding steroid to the incubation mixture in tubes, open to the air, at 37°C in a shaking water bath. In each assay, duplicate incubations were carried out at time 0 and other time intervals up to 7 min; reactions were stopped by the addition of 50 μl 1 N HCl followed by a 3 ml dichloromethane. A 1 μg aliquot of 19-nortestosterone was added to each sample to serve as internal standard by which recovery of steroids could be measured. Steroids were extracted into dichloromethane and the extract evaporated to dryness under a stream of nitrogen. The residue was taken up in 100 μl of 70% methanol and an aliquot subjected to analysis by high performance liquid chromatography (HPLC). Separation was achieved on a C_{18} μBondapak column with a Waters HPLC [9, 10] using an isocratic elution with 60% methanol in water.

When the 3β-HSD assay was performed directly on monolayer cells in culture, the cell monolayers were plated in 35 mm plastic dishes. The medium used for the reaction was the mixture of Dulbecco's modified Eagles Medium and Ham's F-12 medium (1:1, v/v) used for cell culture [12]. Initially all dishes, both control and ACTH-treated cells, were washed with 1 ml aliquots of the reaction mixture containing 1 μM (20R)-20-phenyl-5-pregnene-3β,20-diol (20-PPD) as an inhibitor of cholesterol side chain cleavage reaction [15]. The cells were exposed to the second 1 ml aliquot of the media containing 20-PPD for 3–5 min before starting the assay. To test the effectiveness of the inhibitor, reaction blanks were carried out in which control and ACTH-treated cells were exposed to 2 ml of the media containing 20-PPD. The assay mixture consisted of 2.0 ml of media containing 36 μM dehydroepiandrosterone. The dishes were incubated at 37°C for 60 min after which the medium was removed from each dish and frozen. A 1 μg aliquot of 19-nortestosterone was added to each sample as an internal standard. The samples were then extracted with dichloromethane and the extract dried under a stream of nitrogen. The extract was taken up in 70% methanol and subjected to HPLC using an isocratic elution with 60% methanol. To determine protein content of cells, the cells were scraped from each dish after adding 0.5 ml of 0.5 N KOH. Each dish was washed with 0.5 ml distilled water which was added to the KOH and the protein content determined [13].

RESULTS

The conditions for the 3β-HSD assay were established using PMS from both fresh bovine adrenal gland and bovine adrenocortical cells (BAC) cultured for 24 h (Figs 1A and 1B). With pregnenolone as substrate, the rate of progesterone formation was linear with protein concentration, up to 300 μg PMS

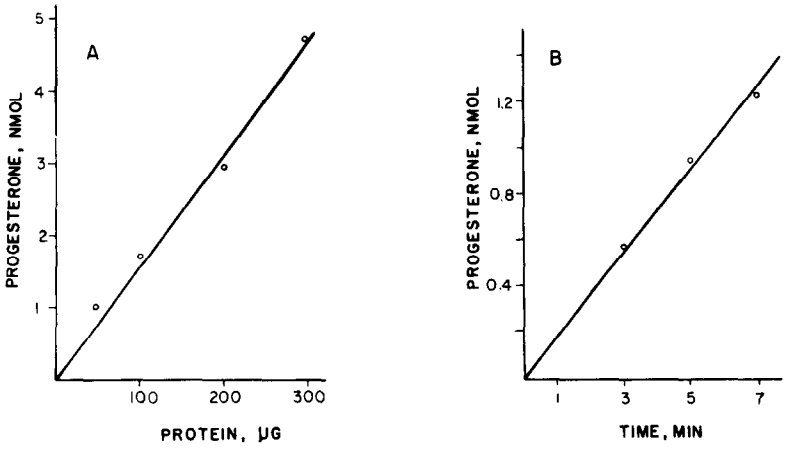

Fig. 1. Effect of protein concentration and time of incubation on the conversion of pregnenolone to progesterone by postmitochondrial supernatant fraction (PMS). 1A: PMS from demedullated bovine adrenal glands; various concentrations of protein incubated with 50 μM pregnenolone for 3 min at 37°C. 1B: PMS from bovine adrenocortical cells maintained 24 h in the absence of ACTH; 150 μg PMS incubated for various time intervals in the presence of 50 μM pregnenolone at 37°C.

from fresh gland, and with time for at least 7 min. The analysis of peaks detected by high performance liquid chromatography showed that the sole product detected was one with the mobility of progesterone (data not shown); the progesterone peak was absent in time zero samples. The 3β-HSD activity (see Tables 1 and 2) in PMS from fresh adrenal gland ranged from 5.08 to 6.20 nmol/min/mg PMS protein. Dispersion of adrenocortical cells by enzymatic digestion did not lower the 3β-HSD activity; however, in confluent cultured cells, the 3β-HSD activity was some 4 times below that noted in PMS from fresh gland (Table 1). The presence of ACTH (1×10^{-6} M) in cell cultures for 24 h failed to alter the 3β-HSD activity; the levels in both control and ACTH-treated cells were virtually the same (Table 1).

In subsequent experiments (Table 2) both the 3β-HSD and 17-OHase activities were measured in preparations from cells maintained for the various times in the presence or absence of ACTH. Exposure of BAC to ACTH for 40 or 48 h produced a marked increase in the 17-OHase activity in PMS isolated from those cells (Table 2). In those same preparations, however, the conversion of pregnenolone to progesterone was unaffected by the prior exposure of the culture to ACTH.

In a separate experiment (Table 2), BAC had been cultured for 46 h in the absence or presence of ACTH following which the incubation medium had been removed and the cells frozen in the culture dishes. The dishes were thawed, sucrose-HEPES buffer was added to each dish and the cells removed by scraping. The homogenate from this cell preparation was subjected to enzyme analysis. Marginal 17-OHase activity was detected in homogenate from control cells in contrast to the high activity detected in the preparation from BAC exposed to ACTH. Again there was no difference in the rates of conversion of pregnenolone to progesterone.

The 3β-HSD activity was determined in cell mono-

Table 1. 3β-Hydroxysteroid dehydrogenase (3β-HSD) activity in postmitochondrial supernatant fluid (PMS)

Preparation	3β-HSD activity nmol/min/mg protein
Bovine adrenal gland[a]	5.08
Dispersed adrenal cells[b]	6.52
Cell cultures	
24 h Control[c]	1.23
24 h + ACTH 1 μM[c]	1.13

[a]Rate derived from data illustrated in Fig. 1A where averages from duplicate incubations were used in linear regression calculations from which plot and rate values were obtained.
[b]Average of duplicate incubations for 3 min corrected for time zero blanks.
[c]Rate for 24 h control derived from data illustrated in Fig. 1B where averages from duplicate incubations were used in linear regression calculations from which plot and rate values were obtained. Rate for 24 h + ACTH samples determined from duplicate incubations carried out at 3 different time intervals from which linear regression calculations were obtained.

Table 2. Activities of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17α-hydroxylase (17α-OHase) in preparations from bovine adrenocortical cells in cultures and from bovine adrenal gland

Sample	Treatment	Preparation	Enzyme activity nmol/min/mg protein 3β-HSD	17α-OHase
Expt 1 48 h culture				
	Control	PMS	1.50	0.01
	ACTH	PMS	1.47	0.50
Expt 2 40 h culture				
	Control	PMS	1.74	0.10
	ACTH	PMS	1.87	2.05
Expt 3 40 h culture				
	Control	Homogenate	0.37	0.02
	ACTH	Homogenate	0.42	0.89
Bovine glands		PMS	6.20	0.60

Table 3. Conversion of exogenous Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids by monolayer cultures of bovine adrenocortical cells

Treatment	Pre-incubation cortisol[a] μg/24 h/dish	Precursor[b]	Δ^4-3-Keto steroid products[c] nmol/h/mg protein
Expt 1			
36 h culture			
Control cells	0.78	17-OHPe	10.3 ± 2.92 (6)
ACTH 1 μM	27.40	17-OHPe	8.2 ± 2.10 (5)
Expt 2			
36 h culture			
Control cells	0.95	DEA	14.5 ± 2.11 (3)
ACTH 1 μM	14.60	DEA	18.9 ± 4.08 (4)

[a]Samples of media for each BAC group were obtained by taking an aliquot of media from each dish as the preincubation medium was removed. Aliquots were pooled and the cortisol content determined by radioimmunoassay. From the cortisol content (μg/ml) a value was determined to indicate the μg formed in the 24 h interval preceding the incubation. Average protein content was 1.10 ± 0.104 for control dishes and 1.35 ± 0.114 for ACTH-treated dishes.

[b]17-OHPe for 17α-hydroxypregnenolone incubated at 56 nmol/dish; DEA for dehydroepiandrostenedione incubated at 72 nmol/dish.

[c]The values in parentheses represent the number of individual incubations for each treatment condition. For each incubation the total Δ^4-3-ketosteroids formed was determined. The rates are reported as the average of those total values ± standard deviation.

layers in 35 mm dishes by adding steroid substrate to the media in the presence of 20-PPD to inhibit endogenous cholesterol side chain cleavage activity. When the cell monolayers were exposed to 28 μM 17α-hydroxypregnenolone, the total Δ^4-3-keto steroids formed by control and ACTH treated cells were found to be essentially the same (Table 3). With dehydroepiandrostenedione as substrate the total amount of Δ^4-3-ketosteroids formed by cultures in 60 min again was found not to be significantly greater in ACTH-treated cells than in control BAC (Table 3). In the ACTH-treated preparations, however, some 65% more of the androstenedione was converted to 11β-hydroxyandrostenedione than in controls.

DISCUSSION

The results of these studies show that in bovine adrenocortical cells (BAC) ACTH regulation of microsomal steroidogenic enzymes is exerted in a specific manner. The adrenal 3β-hydroxysteroid dehydrogenase (3β-HSD)–isomerase activity was unaltered under conditions where cells exposed to ACTH responded to the effects of the tropic hormone (Tables 2 and 3). BAC exposed to ACTH respond by both an increase in corticosteroid output and a shift to favor greater secretion of cortisol [12]. As indicated by the data summarized in Table 3, cortisol released into the media in the 24 h interval prior to the experiment was 7- to 20-fold greater in the ACTH-treated cultures than in control. The index of responsiveness to ACTH is also evident in the rise in 17α-hydroxylase activity seen in the PMS and homogenates from cell cultures treated with ACTH (Table 2). The conditions for cultures of BAC also were identical to those used to demonstrate the induction of synthesis of cytochrome P-450$_{17\alpha}$ and the C_{21}-hydroxylase, cytochrome P-450$_{C21}$ [9–11], thus were ACTH effective in increasing 3β-HSD in BAC it should have been observed. The evaluation of 3β-HSD activity was conducted in a variety of preparations, subcellular fractions, homogenates and intact cell monolayers; in all cases little difference in activity between control and ACTH-treated cells was detected. Thus under conditions where the cytochrome P-450$_{17\alpha}$ responds readily to ACTH, the 3β-HSD remains unaffected. These findings in BAC are similar to responses detected following *in vivo* administration of ACTH to rabbits where there was a marked increase in microsomal 17-OHase with little to no alternation in 3β-HSD or C_{21}-hydroxylase activities [16].

Variations in 3β-HSD responsiveness to ACTH are evident in adrenal cell cultures from several species. In primary cultures prepared from a transplantable mouse adrenal tumor, the 3β-HSD activity was found to be unaffected by ACTH [17). Though O'Hare and Neville [18] found that 3β-HSD remained high in primary cultures of rat adrenocortical cells, Ramachandran and co-workers [19, 20] noted a marked decline in 3β-HSD levels during a 14-day culture of rat adrenal cells. The decline in 3β-HSD levels in those cultures could be reversed by low levels of ACTH with evidence of increased enzyme synthesis and activity [19, 20]. In the rat, hypophysectomy is followed by a decline in total adrenal 3β-HSD as the gland involutes [21]. However, since the specific activity of adrenal 3β-HSD changed modestly, it was concluded that the relative activity could be in the range of the normal gland [21].

In the rat culture system [19, 20] there was a marked decline in 3β-HSD activity over the 14-day culture period which was reversed by exposure to ACTH. In the BAC system used in the present study, the adrenal cells were in culture a maximum of 9–10 days and though there was a 4-fold decline in 3β-HSD activity (Table 1), ACTH at 1 μM concentration for up to 48 h failed to enhance the activity. Though in BAC the activity of 3β-HSD was below that found in fresh gland, the activity was still above that noted for steroid C_{21}-hydroxylase [10]. Also the 3β-HSD activity was well above that for the 17-OHase in control cells and generally the same as that found in ACTH-treated cells. In addition, the level of 3β-HSD activity did not appear to be limiting, as the rate of steroid production from Δ^4-3β-hydroxy-steroid precursors in cell monolayers (Table 3) was well above the rate of steroid secretion noted over a 24 h interval (see cortisol, Table 3; also see [12]).

The exposure of BAC to ACTH results in a differential trophic affect on steroidogenic enzymes of the adrenal microsomal fraction; there is a marked stimulation of the 17-OHase [9,11, 22] with little to no change in the activities of either the steroid

C_{21}-hydroxylase [10, 22] or the 3β-HSD (Tables 1–3). In this study, after BAC are exposed to ACTH the activity of the 17-OHase reaches a level comparable with that of the 3β-HSD (Table 2). That change can permit a redirection in the route of pregnenolone metabolism allowing for greater formation of 17α-hydroxypregnenolone and a potential for increased adrenal androgen formation.

Both 17α-hydroxypregnenolone and 17α-hydroxyprogesterone are substrates for the C_{17} side chain cleavage by the $C_{17,20}$-lyase enzyme leading to androgen formation [23–25]. Depending on the steroid substrate then, the lyase activity would be in competition with either the 3β-HSD or with the C_{21}-hydroxylase. The ACTH stimulated rise in the 17-OHase without marked affects on 3β-HSD or C_{21}-hydroxylase has an additional impact on this consideration. There is strong evidence that in both pig adrenal and pig testis the 17-OHase and $C_{17,20}$-lyase activities reside within a single molecular entity [23–25] and there are data to support the presence of a single substrate binding site. Thus in the bovine adrenal system, the regulation of the 17-OHase without concomitant rise in 3β-HSD or C_{21}-hydroxylase could lead both to an increase in substrate and increase in enzyme for adrenal androgen formation. Though a number of factors can influence adrenal androgen formation, BAC cultured in the presence of ACTH show a marked increase in steroid secretion; by 36 h the secretions consist of nearly equal amounts of adrenal androgen and cortisol [12]. Based on the conditions used in this present culture system we conclude that the 3β-HSD–isomerase in bovine adrenocortical cells is independent of the tropic regulation by ACTH present at levels adequate to stimulate the 17α-hydroxylase and that the difference in responsiveness of the adrenal microsomal enzymes is important in maintaining optimum capacity of the steroidogenic paths in the adrenal cortex.

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