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Hormonal Regulation of the Adrenocortical Cell

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Monolayer cultures of bovine and human adrenocortical cells have been used to study regulation of growth and function. Homogeneous bovine adrenocortical cells exhibit a finite life span of ~60 generations in culture. Full maintenance of differentiated function (steroid hormone synthesis) requires an inducer such as ACTH and antioxidizing conditions. Full induction of differentiated function occurs only when cellular hypertrophy is stimulated by growth factors such as fibroblast growth factor and serum. ACTH and other agents that increase cellular cAMP inhibit replication but do not block growth factor-induced cellular hypertrophy. ACTH and growth factors together result in a hypertrophied, hyperfunctional cell. Replication ensues only when desensitization to the growth inhibitory effects of ACTH occurs.

Cultures of the definitive and fetal zones of the human fetal adrenal cortex synthesize the steroids characteristic of the two zones in vivo. ACTH stimulates production of dehydroepiandrosterone (DHA), the major steroid product of the fetal zone, and of cortisol, the characteristic steroid product of the definitive zone. Prolonged ACTH treatment of fetal zone cultures results in a preferential increase in cortisol production so that the pattern of steroid synthesis becomes that of the definitive zone. The preferential increase in cortisol production by fetal zone cultures results from induction of 3β-hydroxysteroid dehydrogenase, $\Delta^{4,5}$ isomerase activity, which is limiting in fetal zone cells. ACTH thus causes a phenotypic change in fetal zone cells to that of definitive zone cells.

In both bovine and human adrenocortical cells, the principal effect of ACTH is to induce full expression of differentiated function. This occurs only under conditions where growth substances and nutrients permit full amplication.

Key words: adrenocortical, ACTH, FGF, cAMP, fetal zone, replication, regulation, steroidogenesis, antioxidant

The more closely conditions in cell culture reflect those present in vivo, the more likely identified regulatory mechanisms will be relevant to in vivo cell growth and differentiated function. This creates a paradox since it is necessary to know correct in vivo conditions in order to reproduce these in vitro – and such regulatory conditions are those sought using cell culture models. Despite this paradox, significant advances have been made. Notable recent advances include optimization of media [1], isolation of

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potent growth factors [2–5], development of appropriate substrata [6, 7], and establishment of hormonal optima for a number of cell lines [8]. These advances promise to allow culture of pure populations of different normal cell types so that study of physiology under defined conditions will be possible.

Adrenocortical cell cultures have been especially useful for investigation of regulatory mechanisms that control cell growth and function. Adrenocortical cells have a well-defined differentiated function, the production of steroid hormones, and grow in vivo in two well-defined circumstances: 1) following unilateral adrenalectomy without a requirement for the pituitary hormone, adrenocorticotropin (ACTH) [9, 10], and 2) when circulating levels of ACTH are chronically elevated [11–13]. The adult adrenal cortex is organized into three zones which can be distinguished morphologically and/or by the pattern of steroids produced. The production of aldosterone by the outer glomerulosa zone is regulated principally by angiotensin, whereas the production of cortisol and 19-carbon steroids by the inner fasciculata and reticularis zones is regulated by ACTH. In primates, there is a large fetal zone that synthesizes steroid precursors destined for placental metabolism; this fetal zone regresses following delivery and is absent postnatally. Control of growth and function of this zone, which is unique to fetal life and its relationship to the adult cortex, has been a question of interest and importance for development.

We have utilized cultures prepared from bovine and from human fetal adrenal cortices to study regulatory mechanisms. Bovine adrenocortical cells prepared from the fasciculata zone replicate well in culture in F-12 medium and selected lots of fetal calf serum. Although clonal lines have been obtained [14], mass cultures are also homogeneous and consist purely of adrenocortical cells. Several lines of evidence attest to purity [15, 16]. The three most compelling arguments for purity are 1) maintenance of a constant level of induced total steroid production per cell throughout the finite culture life span of ~60 generations; 2) uniform inhibition of cell proliferation by ACTH; and 3) uniform stimulation of replication by angiotensin, which is a specific mitogen for adrenocortical cells. Some characteristics of cultured bovine adrenocortical cells are listed in Table I. It is evident that this endocrine cell is subject to extensive regulation of both growth and function.

Cultures prepared from separated zones of the human fetal adrenal cortex produce steroids that are characteristic for each zone. As discussed below, these cultures have been used to investigate regulation of the function of the specialized fetal zone.

Relationship Between Growth and Differentiated Function

Growth of bovine adrenocortical cells in culture is stimulated by fibroblast growth factor (FGF) and angiotensin; growth stimulated by these agents is augmented by insulin and by insulin-like growth factors (somatomedin C and multiplication stimulating activity) [14, 17, 18]. FGF, which is the most potent growth factor identified, has a number of effects on cultured bovine adrenocortical cells. FGF not only stimulates the rate of proliferation of cells and increases saturation density, but also is required for survival and growth at low cell density necessary for cloning [17]. Although cells initially grow well in fetal calf serum alone, later passage cells require FGF for proliferation [19]. Gospodarowicz et al [20] have indicated that a principal effect of FGF is to stimulate the production of extracellular matrix. When bovine adrenocortical cells are subcultured onto matrix, the requirement for FGF for proliferation is reduced. Angiotensin is a less effective growth stimulator than FGF, but is specific for adrenocortical cells [18]. Angiotensin is of

TABLE I. Characteristics of Cultured Bovine Adrenocortical Cells

Diploid with normal bovine karyotype of 60 telocentric chromosomes.
Cell culture life span of ~60 generations.
Inducible steroidogenic pathway with quantitative maintenance of stimulated steroid production/cell throughout the culture life span.
Growth stimulated by fibroblast growth factor and angiotensin; growth augmented by insulin and insulin-like growth factors.
Steroidogenesis stimulated by ACTH, PGE_1, angiotensin, cholera toxin, and cAMP.
Growth arrest in G_1 induced by ACTH and cAMP.

especial interest because it stimulates both cell proliferation and the differentiated function of steroidogenesis. Both responses exhibit the same dose-response curves, exhibit the same relative responses to angiotensin II precursors, and are inhibited by specific angiotensin antagonists. In addition to acutely stimulating steroidogenesis, angiotensin increases the activity of the steroidogenic pathway, including 11β-hydroxylase [19, 21].

In contrast, ACTH, the major hormone regulator of adrenocortical fasciculata cells, is a potent inhibitor of cell replication [15, 17, 22–24]. ACTH inhibits cell replication with an ED_{50} of 0.1 nM, identical to that required for acute stimulation of steroidogenesis and for induction of 11β-hydroxylase activity [15, 21]. Other agents that increase cellular cAMP concentrations such as PGE_1, cholera toxin, and monobutyryl cAMP similarly inhibit cell replication with the same ED_{50} required for stimulation of steroidogenesis [15, 24]. Cells are arrested in the G_1 phase of the cell cycle.

Both Y-1 mouse adrenocortical tumor cells [25, 26] and normal bovine adrenocortical cells [27], when arrested in G_1 with ACTH or cAMP, are hypertrophied with increased macromolecular content compared either to randomly growing or to serum-deprived cells. In order to explore the effects of ACTH/cAMP, bovine adrenocortical cells that were arrested in G_1 by serum deprivation were stimulated by addition of serum containing FGF in the absence and presence of 8-Br-cAMP. When serum containing FGF was added, there was a 12-h lag prior to the initiation of DNA synthesis (Fig. 1). As determined by flow microfluorometric analysis, 75% of the cells had left G_1 by 20 h and cell division began at 24 h. Median cell volume, which directly correlates with RNA and protein content [25], increased throughout the cell cycle then decreased after 24 h as the cells divided. When 8-Br-cAMP was added together with serum and FGF, initiation of DNA synthesis was inhibited and cells did not divide (Fig. 1). Median cell volume more than doubled and, in the absence of cell division, remained elevated. In the absence of FGF and serum, 8-Br-cAMP did not cause an increase in median cell volume. Therefore, 8-Br-cAMP inhibited DNA synthesis stimulated by serum and FGF, but did not affect the cellular hypertrophy stimulated by these agents. These results obtained in cultured adrenocortical cells indicate that cAMP inhibits the initiation of DNA synthesis by a mode of action different from the depression of the pleiotypic program caused by serum deprivation [28].

To determine the position of arrest in G_1 induced by 8-Br-cAMP, cumulative autoradiography was used. The time course of entry of cells into S following removal of 8-Br-cAMP was compared with that following serum and FGF addition to serum-deprived cells. Following 8-Br-cAMP removal, cells immediately entered S at an exponential rate similar to that observed in serum plus FGF-treated cultures after a 12-h lag [27]. In bovine

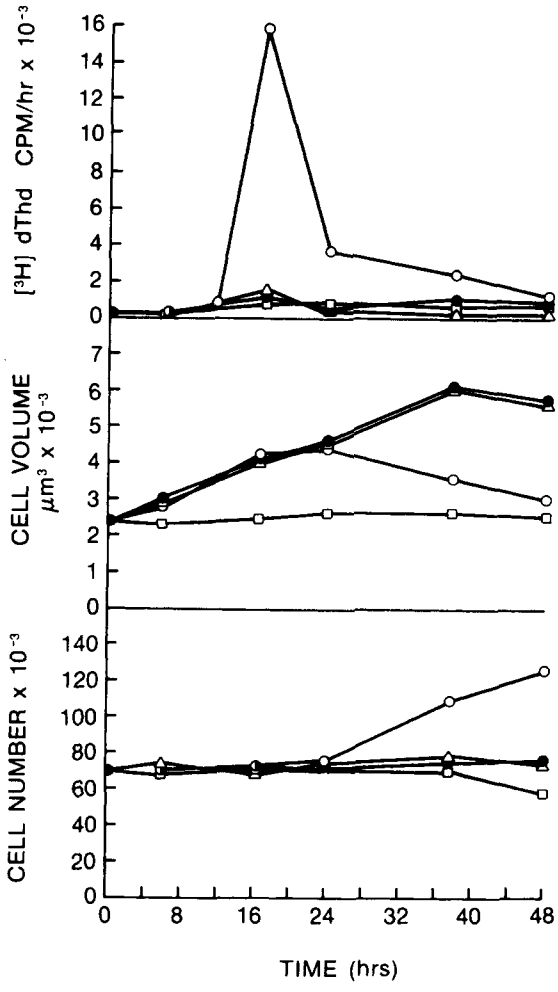

Fig. 1. Effect of 8-Br-cAMP on serum- and FGF-stimulated DNA synthesis, median cell volume, and cell division. Cloned bovine adrenocortical cells were arrested in G_1 by serum deprivation for 72-h. At 72-h, medium was replaced with fresh F-12 without serum (□); 10% FBS and 40 mg/ml FGF (○); 10% FBS, 40 ng/ml FGF and 1.5 mM 8-Br-cAMP (●); or 10% FBS, 40 ng/ml FGF, 1.5 mM 8-Br-cAMP and 0.5 mM theophylline (Δ). At the indicated times, duplicate plates were analyzed for [^{3}H] thymidine incoporation into DNA, cell volume, and cell number. Reprinted from Gill et al, 1980 [27].

adrenocortical cells, the cAMP-induced arrest point is thus near the G_1/S boundary [27]. This contrasts with Y-1 cells where the ACTH and cAMP-induced arrest point is in early G_1, temporally similar to that caused by serum deprivation [26].

Adrenocortical cells that are hypertrophied and arrested in G_1 by treatment with cAMP have an increased steroidogenic capacity (Table II). In order to determine whether the increased steroidogenic capacity was due to cAMP treatment or was a result of cellular hypertrophy itself or arrest in G_1, cells were treated with hydroxyurea, which arrests cells near the G_1/S boundary in a hypertrophied state. When hydroxyurea was added with serum and FGF to serum-deprived cultures, median cell volume increased from 2,200 to 3,800 μm^3 and DNA synthesis began immediately after hydroxyurea removal. As shown in Table II, hydroxyurea-treated cells that are hypertrophied with arrest near the G_1/S

TABLE II. Effect of 8-Br-cAMP on Steroidogenic Capacity of Bovine Adrenocortical Cells

Additions	ng fluorogenic steroid/10^6 cells/6 h
Synchronized by arrest in serum-free medium[a]	
Serum	510 ± 16
Serum plus 8-Br-cAMP (2 mM)	3,551 ± 175[c]
Serum plus hydroxyurea (2.5 mM)	483 ± 8
Serum plus hydroxyurea plus 8-Br-cAMP	5,738 ± 350[c]
Random growth[b]	
Serum	573 ± 10
Serum plus 8-Br-cAMP for 12 h	6,155 ± 85[c]
Serum plus 8-Br-cAMP for 24 h	9,493 ± 279[c]

[a]Cells were arrested in G_1 by serum deprivation for 48 h. At 48 h, media was replenished with F-12 containing 10% FBS and the indicated additions. After 24 h, medium was removed and fresh media containing 10 μg pregnenolone was added for 6 h. Steroidogenic capacity was quantitated by metabolism of pregnenolone to fluorogenic steroids.
[b]Logarithmically growing cells were treated with 2 mM 8-Br-cAMP for the indicated times prior to assay of metabolism of pregnenolone to fluorogenic steroids.
[c]$P < 0.01$ compared to non-cAMP-treated control
Reprinted from Gill et al, 1980 [27].

boundary do not exhibit increased steroidogenic capacity unless cAMP is also added, indicating that the increased steroidogenic capacity is due to cAMP. Serum was necessary for the effects of cAMP on steroidogenesis because addition of cAMP to cells arrested in serum-free medium did not increase steroid production.

In the presence of ACTH/cAMP and mitogens, hypertrophy of adrenocortical cells occurs but initiation of DNA synthesis is inhibited. The steroidogenic pathway is induced by ACTH or agents that increase cellular cAMP. The combined effects of ACTH and mitogens is to produce a hypertrophied hyperfunctional cell. This may be advantageous in vivo in a dynamically responsive system that must vary steroid production in response to a variety of environmental challenges. Cellular hypertrophy is readily reversible, whereas hyperplasia is reversible only by the rate of cell death exceeding the rate of cell generation.

Although the principal response to elevated circulating concentrations of ACTH in vivo is adrenocortical cellular hypertrophy with increased steroidogenic capacity [11, 29, 30], DNA synthesis and content increase with prolonged elevations of ACTH [12, 31]. In culture when bovine adrenocortical cells are exposed to ACTH over longer periods of time, desensitization to the growth inhibitory effects of ACTH occurs [24]. As demonstrated for other peptide hormones, desensitization is specifically caused by active agonists and is not caused by antagonists or by cAMP or by other agents that increase cellular cAMP such as PGE_1. When desensitization occurs, adrenocortical cells replicate in the presence of high concentrations of ACTH although with a population doubling time twice that observed in the absence of ACTH. Prolonged exposure to ACTH therefore allows limited cell replication when mitogens are present. In vitro it is evident that for either hypertrophy or hyperplasia to occur, increased growth factors are required. Because hypertrophy and ultimately hyperplasia occur in vivo in response to ACTH, a link between ACTH and increased delivery of growth factors to adrenocortical cells is implied. Direct growth stimulatory effects of ACTH on adrenocortical cells have not been observed in vitro in any of the well-studied systems [15, 22, 23, 25]. Although it remains

possible that such direct effects of ACTH occur in vivo, studies in vitro imply that the major direct effect of ACTH is stimulation of differentiated function; growth is stimulated by growth factors. The link between ACTH and growth factors in vivo remains obscure. In adrenal growth which follows unilateral adrenalectomy, profound hyperplasia occurs without any requirement for ACTH [9, 10]. Delivery of growth signals appears neurally mediated under these conditions [32].

CONTROL OF DIFFERENTIATED FUNCTION

An important feature of cultured bovine adrenocortical cells is quantitative retention of an inducible steroidogenic pathway throughout the culture life span [15, 19, 24]. However, the pattern of ACTH-stimulated steroids produced is the expected one only in primary cultures (Fig. 2) [19]. It is evident that even in the initial cultures that have undergone three population doublings, there is a relative excess of deoxycortisol (compound S) compared to its product cortisol (compound F). This deficiency of 11β-hydroxylase (cytochrome $P\text{-}450_{11\beta}$), a cytochrome P-450 enzyme localized in the inner mitochondrial membrane [33], is progressive so that later generation cultures and clonal lines fail to produce cortisol. Instead, when stimulated with steroidogenic agents, these cultures produce deoxycortisol and precursor steroids [19]. Although ACTH increases 11β-hydroxylase activity in rat and mouse adrenocortical cell cultures [34, 35], it had no effect on this activity in bovine adrenocortical cell cultures [19].

11β-Hydroxylase activity decayed with a half-life of 40 h in primary cultures (Fig. 3). The decrease in activity was independent of cell division, since a similar rate of fall in activity/plate occurred when cells were grown in 1% as in 10% fetal calf serum (1 vs 5 population doublings) [21]. The decline in 11β-hydroxylase activity was markedly accelerated by treatment with cortisol, the product of the reaction (Fig. 3). The decrease in 11β-hydroxylase activity was dependent on the duration of treatment with cortisol. Direct addition of cortisol to the 1-h assay or addition of ACTH which increased cortisol during the 1-h assay did not affect 11β-hydroxylase activity. Half-maximal effects occurred at 7 μM cortisol when a 24-h treatment was used. Although these concentrations of cortisol are higher than those present in the general circulation, such concentrations are normally present in venous blood from the adrenal cortex [36].

In order to determine the effective molecular structure required for steroid-induced loss of 11β-hydroxylase activity, a series of steroids were added at 30 μM for 24 h to cultures of bovine adrenocortical cells and 11β-hydroxylase activity then measured (Table III). Steroids with an 11β-hydroxyl substitution were most effective. Steroids with no substitution at the 11 position were also effective. However, none of the tested 11α-hydroxylated steroids nor the 11 ketones caused loss of 11β-hydroxylase activity. An exception was dexamethasone, a highly substituted glucocorticoid with an 11β-hydroxyl group, which did not cause loss of 11β-hydroxylase activity. These results indicate that steroids which are products of or which can be converted into products of 11β-hydroxylase induce loss of enzyme activity. Glucocorticoid structure was clearly not required.

The interaction of products or other pseudo-substrates with cytochrome P-450 enzymes has been demonstrated in several cases [37]. Such interactions may result in the release of oxygen-derived free radicals from the cytochrome P-450-pseudo-substrate-oxygen complex owing to the incapacity of the pseudo-substrate to be oxygenated [38, 39]. Both cytochromes directly and various oxygen-derived free radicals may initiate

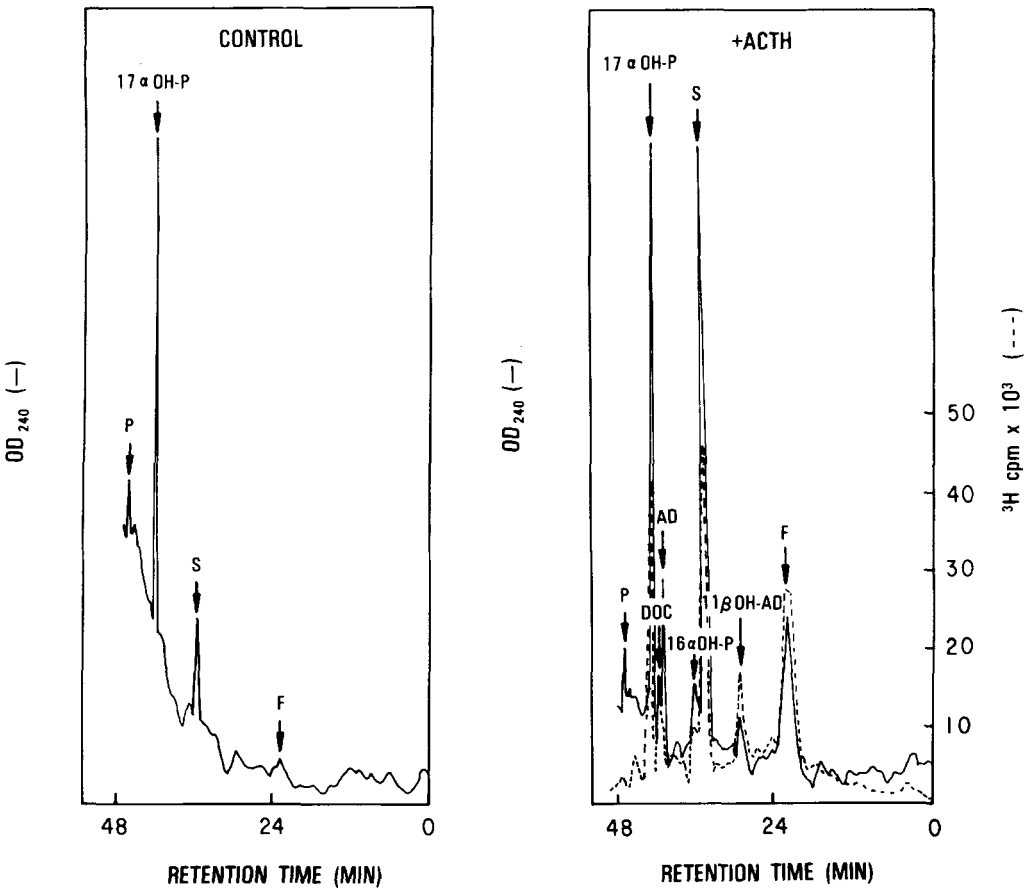

Fig. 2. Effect of ACTH on steroid production in cultured bovine adrenocortical cells. Medium collected from control cultures and from cultures treated with 1 μM ACTH for 24-h was analyzed by high-pressure liquid chromatography. [^{3}H] Pregnenelone was added during the incubation with ACTH and metabolism quantitated from the distribution of radioactivity into each steroid produced (- - -). The retention times of the steroid standards are indicated by the arrows. Reprinted from Simonian et al, 1979 [19].

lipid peroxidation with consequent destruction or inactivation of cytochrome P-450 [40]. The effects of oxygen and antioxidants on cortisol-induced loss of 11β-hydroxylase activity were therefore investigated. Oxygen was used during the treatment period at the standard atmospheric 19% (PO_2 = 140 mm Hg) and at 1% (PO_2 = 7.6 mm Hg). Assays were conducted at 19% O_2. The rate of cell proliferation was equal at both oxygen concentrations (24 h generation times); the lower oxygen concentration of 1% is, in fact, closer to that present in tissue in vivo [41]. As shown in Table IV, the extent of cortisol-induced loss of 11β-hydroxylase is slightly less at 1% than at 19% oxygen. Dimethylsulfoxide (DMSO), a radioprotectant and scavenger of hydroxyl radicals [42], provides significant protection of 11β-hydroxylase activity. Butylated hydroxyanisole (BHA), a terminator of lipid peroxidation chain reactions [43], also prevented cortisol-induced

TABLE III. Effect of Various Steroids on 11β-Hydroxylase Activity in Primary Cultures of Bovine Adrenocortical Cells

	11β-Hydroxylase activity: ng S converted to F/h/mg cell protein				
		Steroid with no. 11 group	11β-OH steroid	11α-OH steroid	11-keto-steroid
No treatment	84.0 ± 3.9				
Deoxycortisol[a]		12.8 ± 1.2	13.0 ± 1.3	84.6 ± 2.5	70.2 ± 2.1
Deoxycorticosterone		16.1 ± 0.1	20.7 ± 0.7	NT	77.6 ± 2.0
Androstenedione		2.9 ± 0.3	27.4 ± 1.7	79.0 ± 4.3	80.1 ± 1.9
Testosterone		6.6 ± 0.3	18.0 ± 1.0	82.9 ± 6.4	86.1 ± 3.6
Dexamethasone			73.8 ± 3.5		
Prednisolone			44.6 ± 4.1		
Estradiol-17β		57.0 ± 5.0			

[a]Parent steroids are listed in the left-hand column. Appropriate substitutions at the 11 carbon position on each steroid are listed above each colume of data. The indicated steroids were added at 30 μM for 24-h prior to quantitation of 11β-hydroxylase activity. Ability to suppress 11β-hydroxylase activity was determined by comparison to control cultures which did not receive steroid. Reprinted from Hornsby, 1980 [21].

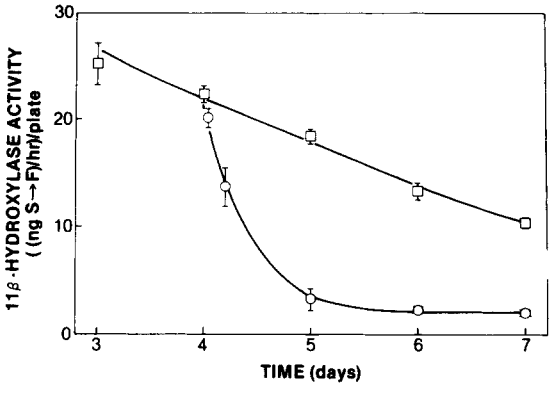

Fig. 3. The effect of cortisol on the half-life of 11β-hydroxylase activity in primary cultures of bovine adrenocortical cells. Four days after plating, one group of plates was treated with medium containing 30 μM cortisol (○) and one group with control medium (□). Equal numbers of cells were present in each plate. 11β-Hydroxylase activity was quantitated by the conversion of [^{3}H] deoxycortisol (S) to cortisol (F) during a 1-h assay incubation. Reprinted from Hornsby, 1980 [21].

loss of 11β-hydroxylase activity. In combination the two antioxidants were completely protective at 1% and highly protective at 19% oxygen. Although ascorbic acid was slightly protective, this did not reach statistical significance; ascorbic acid was, however, significantly protective in human adrenocortical cells. α-Tocopherol was ineffective in both cell types. Antioxidants did not act directly on 11β-hydroxylase because addition during the 1-h assay incubation had no effect. The protective effect of antioxidants was observed only when these were present during the period of treatment prior to the 1-h assay.

TABLE IV. Effect of Antioxidants on Cortisol-Induced Loss of 11β-Hydroxylase Activity

Treatment[a]	11β-Hydroxylase (% activity)	
	1% O_2	19% O_2
None	100	91
Cortisol (30 μM)	35	21
+ BHA (100 μM)	75	55
+ DMSO (10 mM)	84	33
+ BHA (100 μM) and DMSO (10 mM)	100	74

[a]Cortisol (30 μM) was added to primary cultures of bovine adrenocortical cells for 24-h in the presence of the indicated O_2 concentrations and antioxidants. Cells were then washed and 11β-hydroxylase activity was determined during a 1-h assay incubation. Control cultures did not receive cortisol or antioxidant.

Although ACTH or other steroidogenic agents were not able to induce 11β-hydroxylase activity in bovine adrenocortical cell cultures under standard 19% O_2 conditions [16], at 1% O_2 ACTH effectively induced enzyme activity (Fig. 4). The ED_{50} for ACTH induction of 11β-hydroxylase activity of 0.1 nM is similar to that for ACTH stimulation of steroidogenesis. When a range of O_2 concentrations was tested, induction was optimal at 1% O_2. The induction at 1% O_2 was not sustained, however, presumably because of the inhibitory effects of the high levels of cortisol produced (Fig. 5). When DMSO was included, the induction in 1% oxygen was sustained with only a slight fall over a 6-day period.

Cytochrome $P\text{-}450_{11\beta}$ is an inducible cytochrome P-450 species [34, 35], and is subject to inactivation through interaction with 11β-hydroxylated steroids, the products of the enzyme. This inactivation appears to involve lipid peroxidation initiated by oxygen-derived free radicals released from the cytochrome P-450-product-oxygen complex owing to the incapacity of the product to be oxygenated. Maintenance of activity in cell culture therefore requires both inducer (ACTH) and protective antioxidant conditions. The observed susceptibility of steroid metabolizing cytochrome P-450s to lipid peroxidation may explain the active maintenance of antioxidizing conditions in the adrenal cortex. The adrenal cortex has a high concentration and active uptake of ascorbic acid [44], a high concentration of glutathione [45], and concentrates sulfhydryl compounds in general [46]. The maintenance of antioxidizing conditions may be of special importance in steroidogenic tissues which not only produce steroids by cytochrome P-450-mediated reactions but also are exposed to high local concentrations of steroids resulting, for example, from the centripetal blood flow in the adrenal cortex.

Although the steroidogenic pathway is altered during long-term culture under usual conditions of 19% oxygen [19], it remains highly inducible. As shown in Table V, clonal cells that have grown for more than 20 generations in the absence of steroidogenic factors demonstrate low levels of fluorogenic steroid production [19]. Treatment with angiotensin II, cholera toxin, and monobutyryl cAMP increased steroidogenic capacity up to 91-fold. HPLC analysis of steroid products indicated increases in 17α-, and 21-hydroxylase activities as well as in cholesterol side chain cleavage activity. The fluorogenic

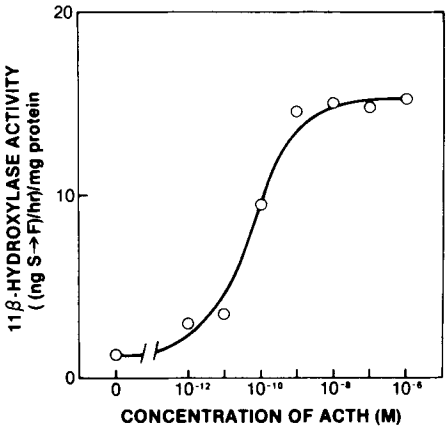

Fig. 4. Induction of 11β-hydroxylase activity by ACTH in bovine adrenocortical cells. On the twelfth day primary cultures were treated for 24-h with the indicated concentrations of ACTH in the presence of 1% O_2. 11β-Hydroxylase activity was then measured during a 1-h assay incubation. Reprinted from Hornsby, 1980 [21].

TABLE V. Fluorogenic Steroid Production by Cloned Bovine Adrenocortical Cells

Additions	Steroid production ($\mu g/10^6$ cells/24-h)
None	0.045 ± 0.009
Angiotensin II (10 nM)	1.6 ± 0.063
Cholera toxin (2.5 nM)	2.3 ± 0.16
Monobutyryl cAMP (1 mM)	4.1 ± 0.21

Confluent cultures were maintained in the presence of the indicated additions for 15 days. Medium was replenished every two days. The 24-h production of fluorogenic steroids was measured in triplicate plates under each experimental condition. Modified from Simonian et al, 1979 [19].

steroids are the 20α-dihydro derivatives of progesterone or 17α-hydroxyprogesterone. Full inducibility is maintained throughout the culture life span of ~60 generations [15, 24].

CONTROL OF DIFFERENTIATION

In primates the fetal adrenal gland, which is very large, is composed of two morphologically and functionally distinct zones. The outer subcapsular zone, which consists of tightly packed small cells with a high nuclear/cytoplasmic ratio, comprises ~20% of the gland [47–49]. This definitive zone is the precursor of the three zones of the adult cortex and synthesizes the same steroids. The inner fetal zone, which consists of large eosinophilic cells, constitutes most of the large fetal adrenal cortex [47, 49–51].

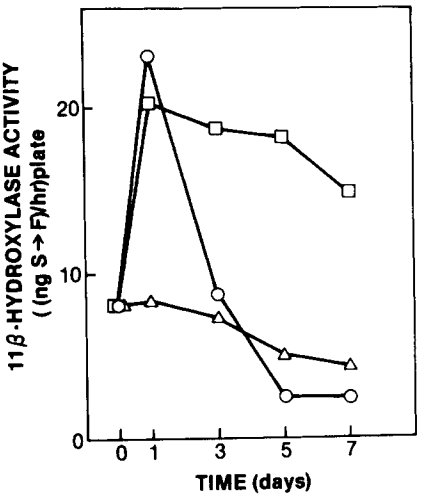

Fig. 5. Induction of 11β-hydroxylase activity by ACTH under varying O_2 and antioxidant conditions. Sixth-day cultures were treated with 1 μM ACTH and 19% O_2 (Δ); 1 μM ATCH and 1% O_2 (○); or 1 μM ACTH and 1% O_2, and 10 mM DMSO (□). 11β-Hydroxylase activity was assayed at the indicated times. Reprinted from Hornsby, 1980 [21].

Cells of this fetal zone have decreased 3β-hydroxysteroid dehydrogenase, $\Delta^{4,5}$-isomerase activity [52, 53]. In the absence of this microsomal enzymatic activity, cortisol, the principal product of the definitive cortex, is not synthesized by the fetal zone [54–56]. Instead, Δ^5-steroids, principally dehydroepiandrosterone (DHA) and its sulfate (DHA-S), are produced in large amounts [53–56]. After birth the fetal zone regresses completely [48, 49, 57]. During fetal life, the Δ^5-steroid products of the fetal zone serve as precursors for placental synthesis of estrogens [58]; cortisol produced by the definitive zone is responsible for a variety of effects in the fetus, including maturation of a number of organs [59].

It is uncertain how the fetal zone of the primate adrenal cortex originates and what hormones stimulate its function. Studies in anencephalic fetuses indicate that the fetal pituitary is required for maintenance of the fetal zone of the adrenal cortex after mid-gestation [60]; the observation that the fetal zone undergoes complete regression post-partum has prompted speculation on the possible role of placental products for maintenance of the fetal zone.

To initiate studies of regulation of the fetal zone of the adrenal cortex, adrenal glands from aborted fetuses of ~16 cm crown-rump length were divided by microdissection into definitive and fetal zones [21]. With the procedure used, definitive zone cultures were free of fetal zone cells; fetal zone cultures, however, contained ~10% definitive zone cells [61]. Cultures from both zones were largely free of fibroblast-like cells. Eighty to ninety-five percent of cells exhibited a marked retraction response to ACTH. This retraction response is specific for adrenocortical cells because cultures of human fibroblasts are flattened and do not respond to ACTH. As with bovine adrenocortical cells, both the rate of proliferation and the saturation density were increased by FGF. ACTH inhibited serum- and FGF-stimulated cell proliferation [61].

Function was assessed by quantitating cortisol (F), the principal product of the definitive zone, and the total of DHA and DHA-S (DHA/S), the principal products of the fetal zone. In fetal zone cultures, ACTH stimulated production of DHA/S and cortisol with a ratio of DHA/S to cortisol of ~4:1 at 15-h (Fig. 6). In definitive zone cultures, ACTH increased production of both steroids with a ratio of 1:1 to 0.8:1. These results with definitive zone cultures agree with those of Branchaud et al [55], who reported that definitive zone cells, like the adult cortex, produce significant amounts of Δ^5-steroids. After 15 h treatment the rate of cortisol production in both cultures increased, suggesting a change in the steroidogenic pathway.

Sensitivity of DHA/S and cortisol production to stimulation was quantitated after 10 h treatment with varying concentrations of ACTH. This 10-h time point precedes the change in slope of steroid production noted in Figure 6. As shown in Figure 7, half-maximal stimulation of steroidogenesis in both zones occurred at ~0.7 nM ACTH. Fetal zone cultures preferentially synthesized DHA/S, whereas definitive zone cultures preferentially synthesized cortisol.

With prolonged exposure to ACTH, fetal zone cultures acquired characteristics of definitive zone cells (Fig. 8). This occurred in the absence of cell division and of cell death. The capacity of fetal zone cells to synthesize DHA/S increased ~2-fold, but the ability to synthesize cortisol increased at least 6-fold so that by day 4 equal quantitites of both steroids were produced. A similar response in fetal zone cultures occurred with treatment with 8-Br-cAMP (1 mM) and cholera toxin (10 nM) (Fig. 9). DHA/S and cortisol production both increased progressively, but cortisol production increased preferentially so that by days 3 and 4 the DHA/S:cortisol ratio was < 1. Although the absolute amounts of each steroid produced by cultures from each zone varied with the cell preparation (Figs. 8 and 9), in all cases prolonged treatment of fetal zone cultures with steroidogenic agents resulted in preferential induction of cortisol production so that the DHA/S:cortisol ratio became < 1, similar to that of definitive zone cultures. Definitive zone cultures increased steroidogenic capacity in response to ACTH with ~ equal quantities of both steroids being produced.

These studies indicated that ACTH induced the activity of 3β-hydroxysteroid dehydrogenase, $\Delta^{4,5}$-isomerase in fetal zone cultures so that the steroidogenic pathway characteristic of definitive zone and adult cortex was acquired. This enzyme activity was assayed directly by quantitating the ability of fetal and definitive zone cells to convert DHA to androstenedione. Controls were similar to those utilized in assessment of 11β-hydroxylase activity [21]. As shown in Table VI, 3β-hydroxysteroid dehydrogenase, $\Delta^{4,5}$-isomerase activity in fetal zone cultures was ~3-fold less than that present in definitive zone cultures. Treatment of fetal zone cultures for 48 h with ACTH resulted in a 5- to 6-fold increase in 3β-hydroxysteroid dehydrogenase, $\Delta^{4,5}$-isomerase activity. The increase in enzyme activity correlated well with the increase in cortisol production in ACTH-treated fetal zone cultures (Figs. 8 and 9).

ACTH thus appears to induce a phenotypic change in fetal adrenocortical cells to that characteristic of definitive and adult adrenocortical cells. Because the fetal zone cultures are initially contaminated with definitive zone cultures to ~10%, the possibility that definitive zone cells account for the preferential increase in cortisol production in fetal zone cultures must be considered. ACTH is growth-inhibitory, and cell number remained constant over the 4-day experiment, making preferential proliferation of definitive zone cultures unlikely. In order to ascribe the increase in cortisol production in ACTH-treated fetal zone cultures to the ~10% contamination with definitive zone cells,

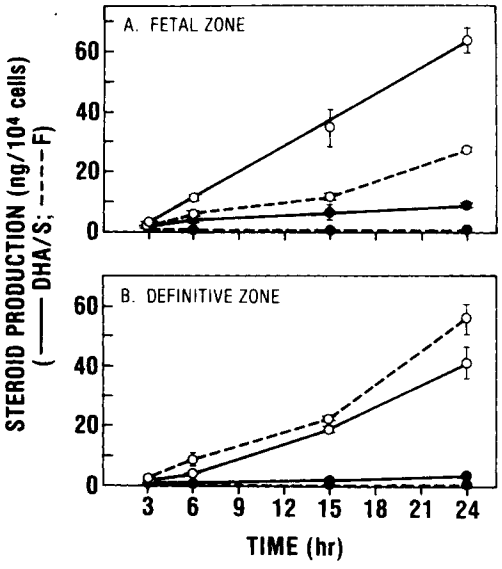

Fig. 6. Effect of ACTH on steroidogenesis in cultures prepared from the fetal and definitive zones of the fetal human adrenal cortex. Media was removed at the indicated times and the cumulative production of DHA/S and cortisol quantitated in aliquots by specific radioimmunoassay. (——), DHA/S; (---), cortisol; (•), minus ACTH; (○), plus 0.1 μM ACTH. Reprinted from Simonian and Gill, 1981 [61].

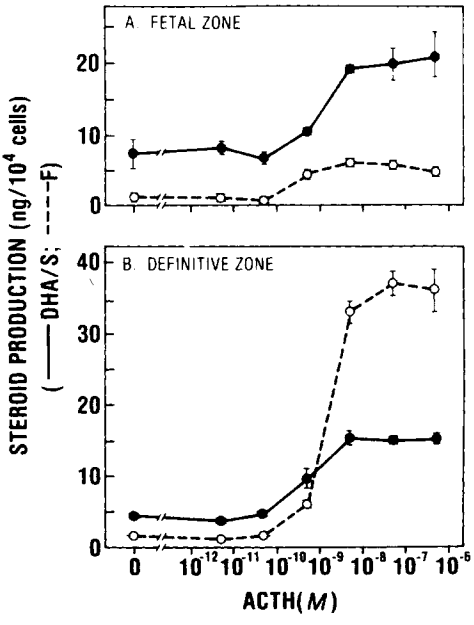

Fig. 7. Effect of varying concentrations of ACTH on steroidogenesis in fetal and definitive zone human adrenocortical cell cultures. After treatment with the indicated concentrations of ACTH for 10-h, medium was analyzed for DHA/S (•) and for cortisol (○) by specific radioimmunoassay. Reprinted from Simonian and Gill, 1981 [61].

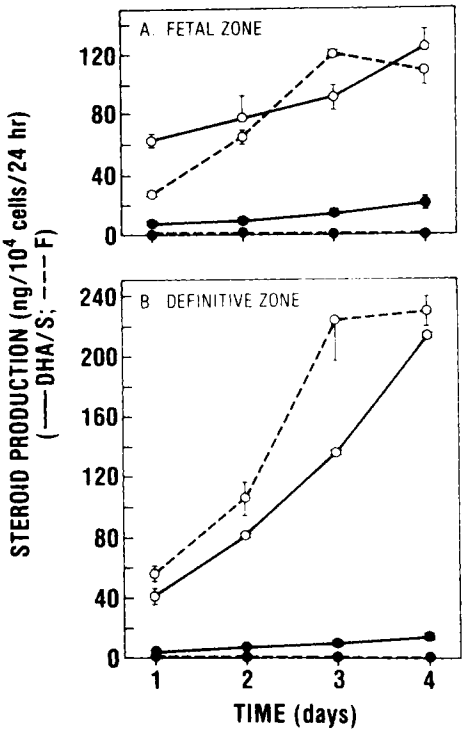

Fig. 8. Effect of prolonged treatment of fetal and definitive zone human adrenocortical cell cultures with ACTH. Medium was renewed each 24-h and DHA/S and cortisol were quantitated. (——), DHA/S; (- - -), cortisol; (●), minus ACTH; (○), plus 0.1 μM ACTH added at each medium change. Reprinted from Simonian and Gill, 1981 [61].

TABLE VI. Effect of ACTH on 3β-Hydroxysteroid Dehydrogenase, $\Delta^{4,5}$-Isomerase Activity in Fetal Zone Cultures

Zone	Additions	Enzyme activity[a]
Experiment 1[b]		
Fetal zone	none	11.3 ± 0.8
Definitive zone	none	28.3 ± 1.1
Experiment 2		
Fetal zone	none	7.6 ± 0.1
Fetal zone	0.1 μM ACTH	41.0 ± 1.0

[a]pmol andostenedione produced/10^6 cells/h. Modified from Simonian and Gill, 1981 [61].

[b]In experiment 1 activities were quantitated on the second day in culture. In experiment 2 ACTH was added on days 7–9 of culture and activity was quantitated on day 10. Similar results were obtained when ACTH was added on day 2. Triplicate cultures were used under each experimental condition.

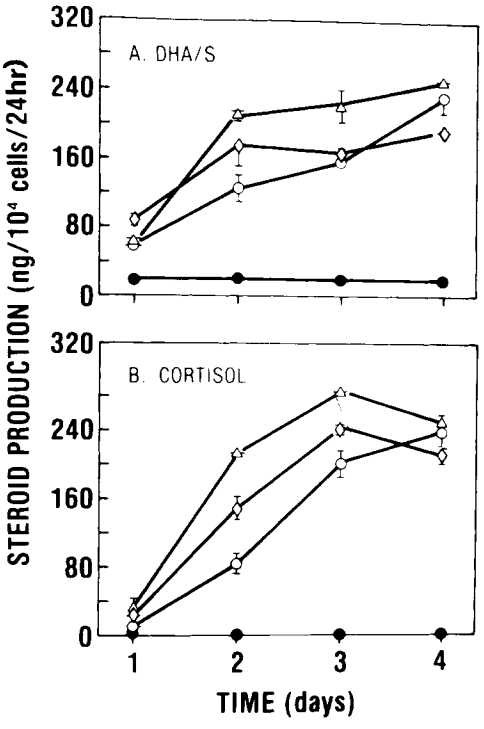

Fig. 9. Steroid production during prolonged treatment of fetal zone cultures with 8-Br-cAMP, cholera toxin, and ACTH. Fetal zone cultures were incubated in the absence (•) or presence of 1 mM 8-Br-cAMP (Δ), 10 nM cholera toxin (◇), or 0.1 μM ACTH (○). Medium was renewed each 24-h and the content of DHA/S (A) and cortisol (B) was determined by radioimmunoassay in triplicate plates at each time point under each experimental condition. Reprinted from Simonian and Gill, 1981 [61].

each definitive zone cell present in fetal zone cultures must produce 5- to 10-fold more cortisol than the equivalent cell present in definitive zone cultures. It thus appears unlikely that the increase in 3β-hydroxysteroid dehydrogenase, $\Delta^{4,5}$-isomerase activity of ACTH-treated fetal zone cultures can be ascribed to contamination by definitive zone cells.

As in bovine adrenocortical cells, the principal action of ACTH is to increase differentiated function. Induction of 3β-hydroxysteroid dehydrogenase, $\Delta^{4,5}$-isomerase in fetal cells produces the definitive/adult cell phenotype. By inference this action of ACTH is prevented in the fetal zone in vivo. Both placental factors and the concentration gradients present because of the organization of blood flow from capsule to inner zones may be important in maintenance of the unique structure and function of the fetal zone.

CONCLUSION

Monolayer cultures of bovine and human adrenocortical cells provide a model system suitable for study of mechanisms that regulate growth and function in vivo. Full expression of differentiated function can be maintained using inducers and antioxidant conditions. Replication is stimulated by growth factors and the relation between repli-

cation and differentiated function has been partially defined. It is evident from these studies that the principal effect of ACTH is to induce full expression of the differentiated function of the adrenocortical cell. This occurs only under conditions where growth substances and nutrients permit full amplification.

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