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Ovine Fetal Adrenal Synthesis of Cortisol: Regulation by Adrenocorticotropin, Angiotensin II and Transforming Growth Factor-β*

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ABSTRACT. An increase in cortisol production by the fetal adrenal cortex is an important prepartum event. The increase in ovine fetal adrenal synthesis of cortisol appears to rely in part on the ACTH induction of 17α-hydroxylase cytochrome P-450 ($P\text{-}450_{17\alpha}$) which occurs before parturition. In the present study we examined the effect of ACTH treatment on cortisol production and $P\text{-}450_{17\alpha}$ expression using primary cultures of ovine fetal adrenal cells. In addition, we examined the effects of angiotensin II (A-II) and transforming growth factor-β (TGFβ) on ACTH-treated cells. We have demonstrated previously that these factors modulate $P\text{-}450_{17\alpha}$ levels in adult ovine and bovine adrenal cells. Fetal ovine adrenal cells were isolated from 126- to 130-day fetuses (term = 144 ± 3 days) and placed in monolayer cell culture. After 1 day in culture the cells were treated with ACTH (10 nM) with or without A-II (0.1–100 nM) or TGFβ (1–100 pM). Medium content of cortisol was low under basal conditions, whereas ACTH-stimulated cortisol production by 10- to 100-fold. A-II and TGFβ inhibited ACTH-induced cortisol production by 70–90%. In addition, 3 days of treatment with ACTH caused a greater than 10-fold induction of $P\text{-}450_{17\alpha}$ enzyme activity in fetal adrenal cells. A-II and TGFβ inhibited the ability of ACTH to induce $P\text{-}450_{17\alpha}$ activity by at least 75%. Using an antibody to $P\text{-}450_{17\alpha}$ and immunoblotting techniques the effects of ACTH, A-II, and TGFβ on enzyme activity were observed to correspond to cellular levels of $P\text{-}450_{17\alpha}$ protein. The inhibitory effects of TGFβ and A-II could not be overcome by the cAMP analog $(Bu)_2$cAMP. Interestingly, the expression of the enzyme 3β-hydroxysteroid dehydrogenase was much less sensitive to inhibition by A-II or TGFβ. The ability of A-II and TGFβ to suppress $P\text{-}450_{17\alpha}$ expression could play a role in determining the pathway of steroidogenesis and specifically the amount of cortisol produced by fetal adrenocortical cells *in vivo*. (*Endocrinology* **129:** 1784–1790, 1991)

IN THE sheep fetus the prepartum surge in plasma cortisol levels is instrumental in the initiation of labor in this species (1, 2). The increase in cortisol may be involved in the maturation of several fetal organs which include the lung, adrenal, and intestine (3). The rise in fetal plasma cortisol appears to be associated with the rapid growth of the fetal adrenal glands (4–7), its greater sensitivity to ACTH (8, 9), as well as an increase in steroid-metabolizing enzymes (10–13). Although the identity of the factors causing the above changes in adrenal differentiation are not firmly established, ACTH appears to play a central role. Indeed, infusion of ACTH into the fetus *in vivo* will, after several days, increase fetal plasma cortisol levels (10–12). An immediate response is not observed because the fetal adrenal cells must increase the levels of steroid-metabolizing enzymes. The enzyme 17α-hydroxylase cytochrome P-450 ($P\text{-}450_{17\alpha}$), in particular, has been shown to be rate-limiting in the production of cortisol by the fetal sheep adrenal (11, 13).

In addition to a role for ACTH in causing differentiation, there also may be factors which inhibit the development of the adrenal and the subsequent rise in cortisol production. Such factors could be of particular importance in species such as the sheep, where an early rise in cortisol could lead to premature labor. Evidence is now accumulating which indicates that certain factors can inhibit the stimulatory effects of ACTH in adrenal cells. We, and others, have shown that two of these factors, angiotensin II (A-II) and transforming growth factor-β (TGFβ), can inhibit the ACTH stimulation of adult bovine and ovine adrenal cell cortisol production (14–19). Fetal sheep plasma levels of A-II are elevated when compared to the mother or nonpregnant adult levels (20, 21). In addition, it has been demonstrated that in the

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mouse fetus expression of TGFβ is high (22). We hypothesize that these factors play a role in modulating fetal adrenal differentiated function. In the present study we use primary cultures of ovine fetal adrenal cells to examine the chronic regulation of cortisol production and the expression of P-450$_{17\alpha}$. We demonstrate that ACTH is a potent activator of both P-450$_{17\alpha}$ expression and cortisol production, whereas A-II and TGFβ can inhibit the stimulatory effects of ACTH.

Materials and Methods

Cell isolation and culture

Fetal ovine adrenals were obtained from 126- to 130-day (term = 144 ± 3 days) twin or triplet fetuses obtained from sheep euthanized with 50 mg/kg sodium pentobarbital. The protocol for this nonsurvival surgery was approved by the Institutional Review Board for Animal Research at the University of Texas Southwestern Medical Center. Fetal ovine adrenocortical cells were prepared by successive treatments with trypsin (0.25%) in Dulbecco's modified Eagle's medium and Ham's nutrient mixture F12 (Sigma, St. Louis, MO) containing antibiotics as previously described. Cells were placed in monolayer culture for 24 h in the same medium containing insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenous acid (6.25 ng/ml), BSA (1.25 mg/ml), and linoleic acid (5.35 μg/ml) added in the form of 1% ITS-plus Premix (Collaborative Research, Bedford, MA). Treatments were started on the second day of culture in this defined medium. Medium containing the various treatments were changed every 24 h until completion of the experiment. ACTH (Cortrosyn) was obtained from Organon (West Orange, NJ), A-II (human), saralasin, and (Bu)$_2$cAMP from Sigma. Porcine TGFβ-1 was obtained from R&D Systems (Minneapolis, MN).

Enzyme, steroid, and cAMP measurement

Steroid 17α-hydroxylase activity was examined in intact cells by measuring the fractional conversion of [^{14}C]progesterone (NEN-DuPont, Boston, MA) to 17α-hydroxyprogesterone and deoxycortisol. Etomidate (1 μM) was included in the incubation medium to inhibit 11β-hydroxylase activity, thereby preventing cortisol or corticosterone accumulation (16, 17). Cells were incubated for 2 h in medium containing approximately 30,000 dpm/ml [^{14}C]progesterone and 20 μM radioinert progesterone. The medium (1 ml) was then extracted with methylene chloride (10 ml). The extracts were dried under a stream of nitrogen. TLC analysis was accomplished using Silica Gel 60 F254 plastic-backed plates (EM Science, Gibbstown, NJ), which were developed using chloroform-ethylacetate (9:1 vol/vol). Progesterone, 17α-hydroxyprogesterone, deoxycorticosterone, corticosterone, deoxycortisol, cortisol, and androstenedione were used as chromatographic standards. Steroid 17α-hydroxylase activity was expressed as nanomoles of 17α-hydroxyprogesterone plus deoxycortisol produced per mg cell protein in 2 h.

Protein immunoblotting

After treatments, cells were washed with 0.9% NaCl solution and removed in a small vol of lysing buffer as previously described (16, 17). One-dimensional electrophoresis was performed in a 12% polyacrylamide gel using a Precast gel system obtained from Amersham (Arlington Heights, IL). Sample buffer contained 0.05 M Tris (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, and 8 mM EDTA, and samples were loaded after boiling for 3 min in the presence of 2-mercaptoethanol. Electrophoresis was carried out at 30 V at room temperature. Proteins were transferred to a nitrocellulose paper in a Transphor electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA), at 100 V for 1 h at 2–4 C in a buffer containing 20% methanol, 20 mM Tris, and 150 mM glycine. The nitrocellulose membrane was incubated for 15 min at room temperature in a blocking buffer containing 10 mM Tris, pH 7.4, 0.15 M NaCl, 0.2% Nonidet P-40, and 0.5% dry milk. The membranes were then placed in the same buffer containing polyclonal antibodies [10 μg immunoglobulin G/ml against human placental 3β-hydroxysteroid dehydrogenase (3βHSD) or pig testis P-450$_{17\alpha}$] for 2 h. Membranes were washed in the same buffer, without antibody, for 15 min, and incubated in the blocking buffer containing approximately 10^6 cpm/ml [^{125}I] protein A (ICN Biochemicals, Irvine, CA) for 30 min. Finally, the nitrocellulose membrane was washed in blocking buffer for 1 h before exposure to photographic film. All incubations and washings were performed at room temperature.

Results

Cortisol production

The effects of A-II and TGFβ on fetal ovine adrenal cell steroid production were determined by measuring medium content of cortisol. Fetal ovine adrenal cells were treated for 1–3 days with medium and factors renewed each day. ACTH (10 nM) treatment lead to an increase in cortisol release by up to 100-fold above basal levels (Fig. 1 and Table 1). ACTH stimulation of cortisol production exhibited a progressive increase with each day of treatment. Treatment of fetal adrenal cells with A-II (100 nM) did not increase cortisol release above basal values (Table 1). However, when A-II was included with ACTH in the experimental medium, the amount of cortisol production was inhibited on each of the 3 days examined (Fig. 1). Relative A-II inhibition was greatest on day 1 (≃96%) with decreased effectiveness by the second (≃84%) and third days of treatment (≃62%). A-II inhibition of cortisol production occurred in a concentration-dependent manner (Fig. 2). The inhibition of cortisol production was readily observed at a concentration of 1 nM A-II, whereas maximal inhibition was seen at doses of 10 nM or higher. The effect of A-II was specific since the A-II antagonist, saralasin (10–1000 nM), was able to overcome the inhibitory effects of A-II (100 nM) in a dose-dependent fashion (Fig. 3). Saralasin by itself did not stimulate or inhibit cortisol secretion (data not shown). In addition, the inhibitory effect of A-II on cortisol production was seen when (Bu)$_2$cAMP (1 mM) was used to stimulate cortisol production (Table 1).

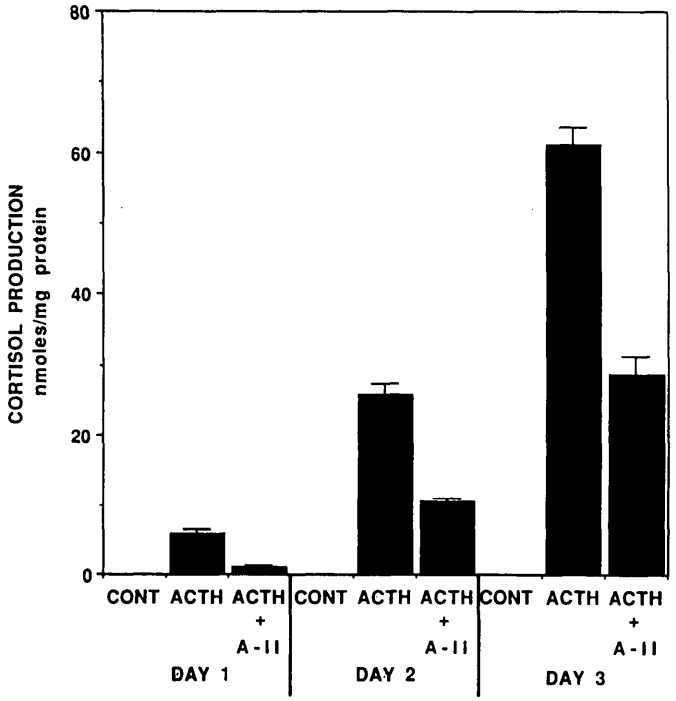

FIG. 1. The effect of A-II on ACTH stimulation of fetal adrenal cortisol production. Fetal adrenal cells were treated for 3 days in the absence or presence of ACTH (10 nM) and A-II (100 nM). Medium and factors were changed each day. Values represent the mean ± SD for four dishes of cells and are representative of three independent experiments.

TABLE 1. Regulation of cortisol production in fetal ovine adrenal cells

Treatment	Cortisol production (nmol/mg protein·24 h)		
	None	A-II (100 nM)	TGFβ (80 pM)
Basal	0.34 ± 0.05	0.43 ± 0.16	0.20 ± 0.04
ACTH (10 nM)	16.22 ± 1.98	3.74 ± 0.40	4.45 ± 0.18
Bu_2cAMP (1 mM)	10.28 ± 1.09	3.13 ± 0.49	2.90 ± 0.54

Fetal ovine adrenal cells were rinsed and treated as described in a defined medium for 24 h. Medium content of cortisol was determined by immunoassay and standardized to the amount of cellular protein. Values represent the mean ± SD for four different dishes of cells.

Similar experiments to those described above were carried out to examine the effects of TGFβ on fetal adrenal cortisol production. Treatment of fetal adrenal cells with TGFβ (80 pM) decreased the already low level of cortisol production (Table 1). In addition, TGFβ almost completely inhibited the ability of ACTH to stimulate cortisol production during the 3-day treatment period (Fig. 4). The inhibitory effect of TGFβ on the production of cortisol was concentration dependent (Fig. 5). An inhibition of cortisol production was observed using a concentration of 1 pM TGFβ, whereas maximal inhibition was seen between 10–40 pM. At the higher concentrations, TGFβ (40–100 pM) inhibited ACTH-stimulated cortisol production by 70–90%. $(Bu)_2$cAMP stimulation of cortisol production was inhibited by TGFβ in a similar manner (Table 1).

Steroid 17α-hydroxylase activity

To determine the effect of A-II and TGFβ on fetal ovine adrenal cell activity of P-$450_{17\alpha}$, we examined the fractional conversion of [^{14}C]progesterone to 17α-hydroxyprogesterone and deoxycortisol. Treatment of cells with ACTH for 3 days increased the amount of P-$450_{17\alpha}$ activity by more than 10-fold (Fig. 6). This increase in enzyme activity could be mimicked by substituting $(Bu)_2$cAMP (1 mM) for ACTH. A-II (100 nM) inhibited the ability of ACTH and $(Bu)_2$cAMP to induce P-$450_{17\alpha}$ activity by greater than 75%. If TGFβ (80 pM) was included in the experimental medium with either ACTH or $(Bu)_2$cAMP the induction of P-$450_{17\alpha}$ was also abolished (Fig. 6).

P-$450_{17\alpha}$ enzyme levels

To examine the effect of ACTH, A-II, and TGFβ on the cellular content of P-$450_{17\alpha}$ enzyme, aliquots of cell protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and P-$450_{17\alpha}$ was visualized after immunoblotting. The level of P-$450_{17\alpha}$ in untreated cells was undetectable, whereas ACTH treatment for 3 days caused the appearance of a prominent band with an approximate mol wt of 57K (Fig. 7). A-II (1–1000 nM) decreased the amount of this immunoreactive band in a dose-dependent manner. Concentrations of A-II greater than 10 nM almost completely blocked the ability of ACTH to induce P-$450_{17\alpha}$ expression. In addition, we examined the level of the steroid-metabolizing enzyme 3βHSD. ACTH increased the amount of immunodetectable 3βHSD severalfold (Fig. 7). When A-II was included in the incubation medium with ACTH, the induction of 3βHSD was blunted but not to the degree seen for P-$450_{17\alpha}$. TGFβ was also able to inhibit the ACTH-induction of P-$450_{17\alpha}$ enzyme levels (Fig. 8). The inhibitory effects of TGFβ occurred in a concentration-dependent manner with concentrations 40 and 100 pM inhibiting P-$450_{17\alpha}$ expression to nearly nondetectable levels. The induction of 3βHSD levels were also decreased by TGFβ (Fig. 8), but not to the extent seen for P-$450_{17\alpha}$.

Discussion

The adrenal glands of the ovine fetus follow an interesting course of biochemical development. By 50 days of a 144-day gestation the ovine fetal adrenal appears functional by the ability to produce cortisol and by its responsiveness to ACTH treatment. Interestingly, responsiveness to ACTH and the production of cortisol are decreased by day 100 of gestation but return by term (9). The ability to produce cortisol appears to follow P-$450_{17\alpha}$ expression within the fetal adrenal (10–13). The present study demonstrates that ACTH is a potent activator of

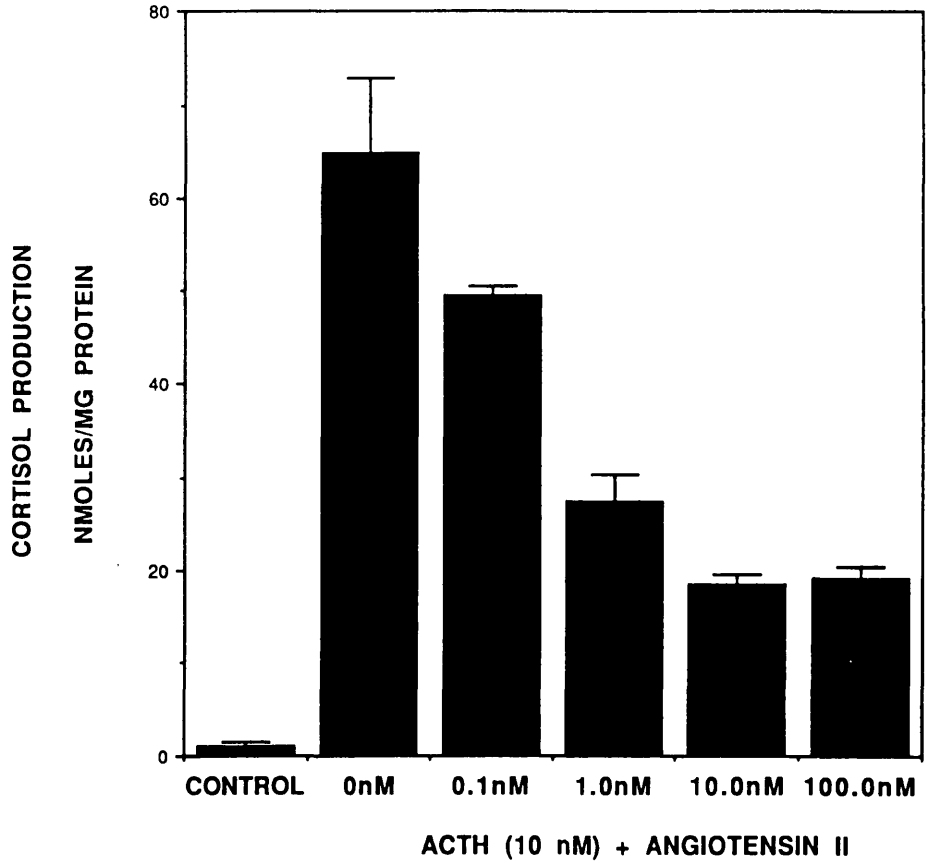

FIG. 2. Concentration dependency of A-II inhibition. Fetal adrenal cells were treated for 24 h alone or with ACTH (10 nM) and increasing concentrations of A-II. Values represent the mean ± SD for cortisol content of the medium from four separate dishes of cells and are representative of three independent experiments.

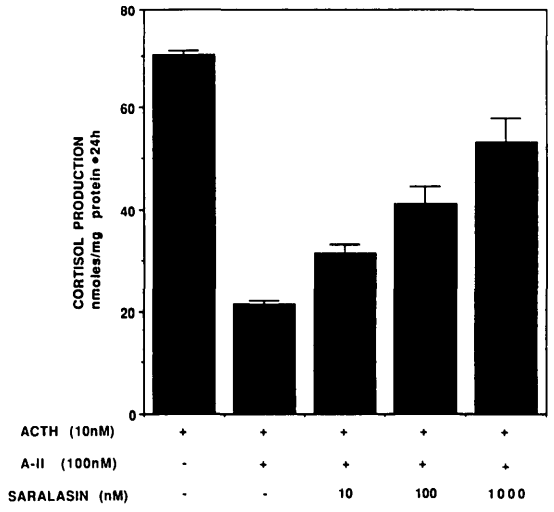

FIG. 3. Effect of saralasin on A-II inhibition. Fetal adrenal cells were treated for 24 h in the absence or presence of ACTH (10 nM), A-II (100 nM), and increasing concentrations of the A-II antagonist, saralasin. Values represent the mean ± SD for cortisol content of the medium from four separate dishes of cells.

P-450$_{17\alpha}$ expression and cortisol production in ovine fetal adrenal cells *in vitro*. In addition, we show that A-II and TGFβ are able to inhibit the ability of ACTH to induce both cortisol production and P-450$_{17\alpha}$ expression.

Both *in vitro* and *in vivo* studies have shown that ovine fetal adrenal maturation is induced by ACTH administration (10–12). ACTH increases the coupling of the ACTH receptor to adenylate cyclase resulting in a stimulation of cAMP production by the fetal adrenal cells (8, 9). ACTH treatment *in vitro* and *in vivo* also increases P-450$_{17\alpha}$ expression (10–12). Our data extends the previous reports by examining the ability of ACTH to induce the levels of P-450$_{17\alpha}$ and 3βHSD protein in fetal sheep adrenal cells. The level of P-450$_{17\alpha}$ protein paralleled the ACTH effect on P-450$_{17\alpha}$ activity. Therefore, fetal sheep adrenal cells increase expression of P-450$_{17\alpha}$ and 3βHSD protein presumably via an increase in messenger RNA accumulation, a manner similar to that we have seen in adult bovine and ovine adrenal cells (17, 23).

In addition to the potent stimulatory action of ACTH, it has been suggested that in the sheep fetus there are endogenous inhibitors of adrenal differentiation. This hypothesis is based on the observation that when placed in primary culture, ovine fetal adrenal cells spontaneously exhibit increased responsiveness to ACTH and steroidogenic capacity (10). Using adult bovine and ovine adrenal cells in primary culture, we recently demonstrated that A-II and TGFβ are potent inhibitors of cortisol production (16–17). TGFβ appears to affect adrenal cell function at several sites beginning with a rapid suppression of steroid production which occurs during

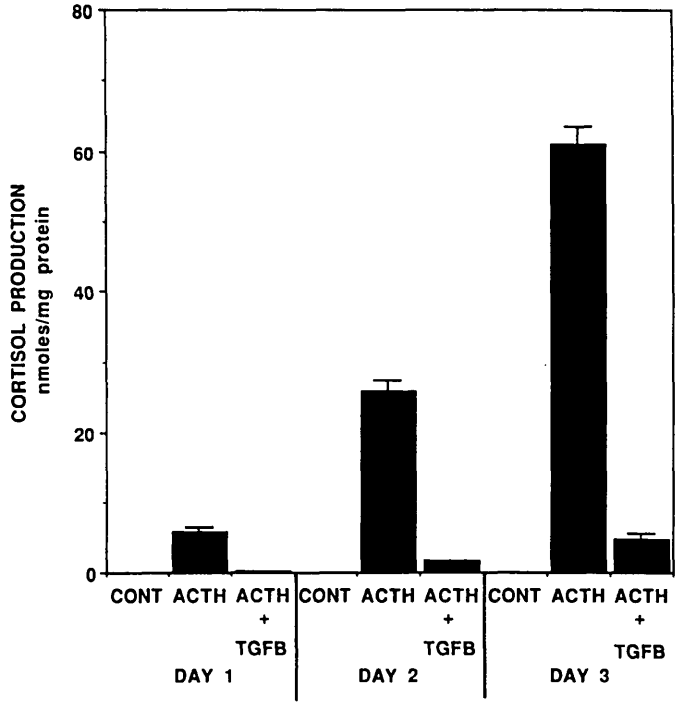

FIG. 4. The effect of TGFβ on ACTH stimulation of fetal adrenal cortisol production. Fetal adrenal cells were treated for 3 days in the absence or presence of ACTH (10 nM) and TGFβ (80 pM). Medium and factors were changed each day. Values represent the mean ± SD for four dishes of cells and are representative of three independent experiments.

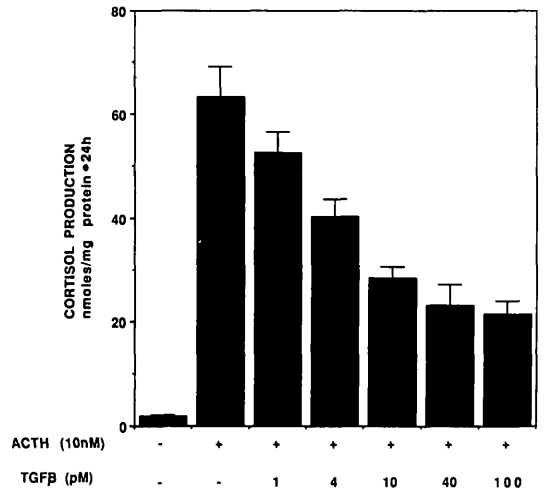

FIG. 5. Concentration dependency of TGFβ inhibition of cortisol production. Fetal adrenal cells were treated for 24 h alone or with ACTH (10 nM) and increasing concentrations of TGFβ. Values represent the mean ± SD for cortisol content of the medium from four different dishes of cells.

the initial 12 h of TGFβ treatment. Chronic treatment with TGFβ also decreases ACTH and low density lipoprotein (LDL) receptor number as well as expression of steroid-metabolizing enzymes (14, 16–18). In the current study, we show that both A-II and TGFβ are able to inhibit the majority of cortisol production within the

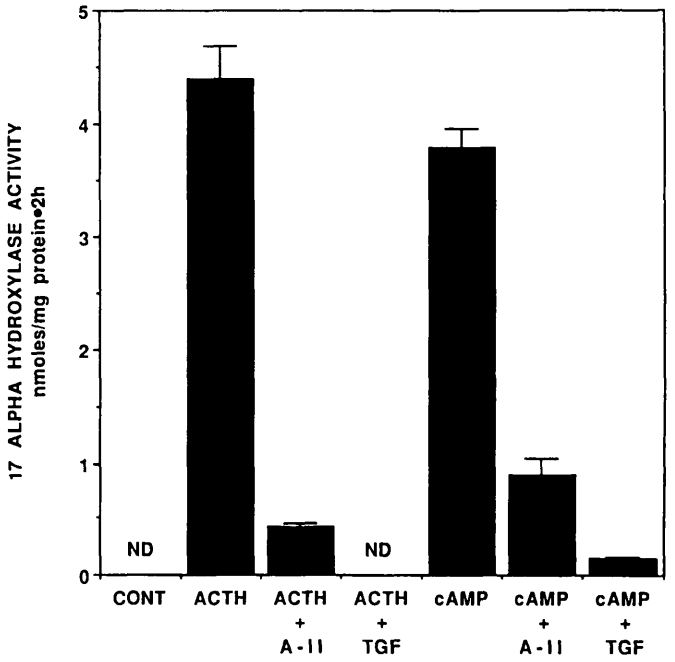

FIG. 6. Regulation of fetal adrenal cell steroid 17α-hydroxylase activity. Cells were treated for 3 days in the absence or presence of ACTH (10 nM), A-II (100 nM), TGFβ (80 pM), and $(Bu)_2$cAMP (cAMP 1 mM). At the end of treatment cellular 17α-hydroxylase activity was examined. Values represent the mean ± SD for triplicate samples from a representative experiment. ND, Nondetectable.

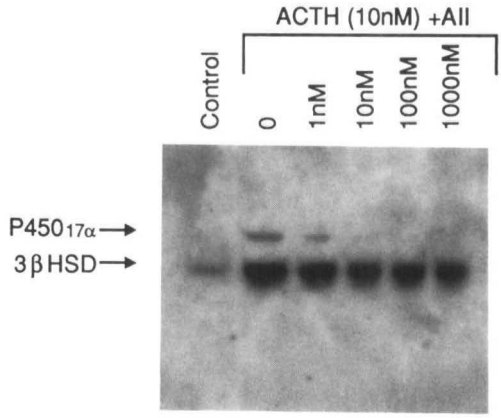

FIG. 7. Regulation of P-$450_{17\alpha}$ and 3βHSD protein expression by A-II. Cells were treated for 3 days in the absence or presence of ACTH (10 nM) and increasing concentrations of A-II. At the end of treatment 75 μg total cell protein was used for immunoblotting analysis.

first 24 h of treatment. The inhibition of cortisol production was not due to its effects on the use of low density lipoprotein cholesterol since all experiments were conducted in a defined medium which was lipoprotein-free. In addition, the inhibition of $(Bu)_2$cAMP-stimulated cortisol production suggests that an effect of TGFβ on fetal adrenal cell ACTH receptors also was not solely responsible. Thus, like in the adult ovine and bovine adrenal, TGFβ appeared to act at multiple levels within the cell.

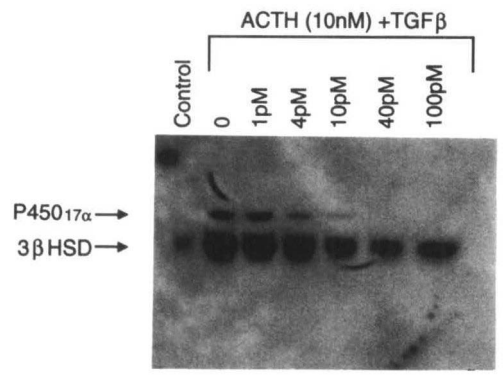

FIG. 8. Regulation of P-450$_{17\alpha}$ and 3βHSD protein expression by TGFβ. Fetal adrenal cells were treated for 3 days in the absence or presence of ACTH (10 nM) and increasing concentrations of TGFβ. At the end of treatment 75 μg total cell protein was used for immunoblotting analysis.

Another chronic effect of A-II and TGFβ on cultures of ovine fetal adrenal cells was the inhibition of ACTH expression of P-450$_{17\alpha}$ and 3βHSD. Importantly the expression of P-450$_{17\alpha}$ was decreased to a greater degree. These data suggest that differential regulation in the expression of these enzymes may occur. Such a case has been observed in human fetal adrenal (19) and adult bovine adrenal cells (18). The differences in these enzymes are of particular importance when one considers the adrenal zones and the secretion of zone-specific steroids. We recently have shown that ovine fetal adrenal cells are capable of producing aldosterone in response to A-II (24). The ability of A-II to inhibit expression of P-450$_{17\alpha}$ and increase aldosterone production furthers the hypothesis that this hormone is involved in the regulation of adrenal zonation.

Although the inhibitory effects of TGFβ and A-II appear similar in the fetal adrenal cells, the reported intracellular mechanism of action for these peptides does not seem to be the same. In adult adrenal cells A-II activates the hydrolysis of membrane phospholipids yielding the two second messengers diacylglycerol and inositol trisphosphate. Diacylglycerol proceeds to activate protein kinase C, and inositol trisphosphate causes the release of intracellular calcium stores (reviewed in Ref. 25). The activation of protein kinase C by A-II appears to be responsible for the inhibition of P-450$_{17\alpha}$ expression in fetal human adrenal cells and adult bovine adrenal cells (18, 19). The exact intracellular mechanism of action for TGFβ is not currently known.

The regulatory role of A-II or TGFβ in the fetus is not fully defined. However, levels of A-II circulating in the fetus are greater than in the maternal or nonpregnant sheep circulation (20–21). It is possible that levels of A-II are involved with the development of the renin-angiotensin system and could play a role in initiating adrenal aldosterone production (26). Data in the present study would suggest that the renin-angiotensin system may be involved in a negative modulation of cortisol levels acting directly on adrenal differentiation. The mechanism by which ACTH would overcome the inhibitory effects of A-II and cause the prepartum surge in cortisol remains to be addressed. However, the magnitude of the rise in plasma ACTH levels near term could be involved with decreasing A-II effectiveness.

In conclusion, the results show that ACTH stimulates the production of cortisol and expression of P-450$_{17\alpha}$ protein. The data also show that the ACTH stimulation of fetal adrenal cell cortisol production can be inhibited by A-II and TGFβ. Furthermore, the inhibitory effects of both A-II and TGFβ were manifest at the level of P-450$_{17\alpha}$ expression. These findings provide insight into the mechanisms involved in causing the cortisol surge seen in late gestation within the ovine fetus. In addition, we suggest that A-II and/or TGFβ may be instrumental in preventing a premature increase in cortisol production by the ovine fetal adrenal.

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