Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 7795–7799, October 1986
Cell Biology

Differential effects of transforming growth factor type β on the growth and function of adrenocortical cells *in vitro*

(cell differentiation/fibroblast growth factor/steroidogenesis)

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Communicated by Roger Guillemin, July 16, 1986

ABSTRACT **Transforming growth factor type β (TGF-β) suppresses basal as well as corticotropin (ACTH)-stimulated steroid formation by bovine adrenocortical cells in culture. The effect is dose dependent and is not accompanied by any change in adrenocortical cell growth. The minimum effective dose of TGF-β is 4×10^{-13} M (10 pg/ml), and maximal inhibition is observed at a concentration of 4×10^{-11} M (1 ng/ml). A 16- to 20-hr incubation with TGF-β is required to decrease steroidogenesis, and 12–18 hr are required before cells treated with TGF-β recover complete responsiveness to corticotropin. Increases in cAMP mediated by corticotropin, forskolin, and isobutylmethylxanthine are not modified by the addition of TGF-β; thus adenylate cyclase activity is unaffected by TGF-β. Although TGF-β inhibits the formation of all of the Δ^4-steroids measured (including cortisol, corticosterone, aldosterone, and androstenedione), its effect can be completely reversed by the addition of 25-hydroxycholesterol, pregnenolone, or progesterone to the cells. In contrast, the addition of low density lipoprotein has no effect suggesting that TGF-β targets the conversion of cholesterol precursors to cholesterol. The results demonstrate a highly potent effect of TGF-β on the differentiated function of the adrenocortical cell. The inhibition of steroidogenesis can be dissociated from any effect on cell proliferation, and it occurs distal to the formation of cAMP but proximal to the formation of cholesterol. The results suggest that in the adrenal, TGF-β or TGF-β-like proteins may be playing an important role in modifying the differentiated state of the adrenocortical cell.**

Factors other than corticotropin (ACTH) may be modulating adrenocortical function. These include the observations that the formation of Δ^4- and Δ^5-steroids by the adrenal cortex is differentially regulated in the fetal adrenal (1) and during adrenarche (2). While several naturally occurring substances like angiotensin II (3), γ-melanocyte-stimulating hormone (4), and atrial natriuretic factor (5) can modify the adrenocortical response to ACTH, their contribution to normal growth and function of the adrenal cortex is not fully understood. Perhaps just as important, adrenocortical cells are also highly responsive to growth factors. Basic fibroblast growth factor (FGF), as an example, is the most potent mitogen known for adrenocortical cells (6) and can delay cell senescence *in vitro* (7). This growth factor, originally characterized from the pituitary gland (6), was also isolated and characterized from extracts of the bovine adrenal (8) suggesting that it may play an important function in the local maintenance of adrenocortical function.

Because the results from several clinical and experimental studies have also suggested that there is a significant modification in adrenocortical function during such processes as endotoxemia (9–11), we tested the hypothesis that factors, potentially derived from monocytes, could be directly influencing the adrenocortical response. In this report we describe the potent effects of one of these substances, transforming growth factor type β (TGF-β), to modulate adrenocortical steroidogenesis. This substance, originally isolated on the basis of its capacity to induce anchorage-independent cell growth has been isolated from several tissues and has been shown to have numerous distinct biological activities.

MATERIALS AND METHODS

Cell Culture. Bovine adrenocortical cells were prepared for cell culture as follows: adrenal glands were transported to the laboratory and dissected; the adrenal cortex was minced and washed in a solution of Hepes-buffered bovine serum albumin (12). After a 1-hr incubation with collagenase (4 mg/ml) and DNase (40 μg/ml), the suspension was filtered through cotton gauze and centrifuged. The cell pellet was resuspended in culture medium consisting of Hepes-buffered Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) horse serum and 2.5% (vol/vol) fetal calf serum. Aliquots were either frozen in the presence of dimethylsulfoxide or distributed into 24-well tissue culture dishes. On the second and third days of culture, the cells were washed twice with culture medium, and on the third day the experiments were started by the addition of 10 μl of rat ACTH-(1–39), basic FGF, or TGF-β.

Radioimmunoassays. The radioimmunoassays (RIAs) for cortisol, corticosterone, aldosterone, and Δ^4-androstenedione were performed with antibodies obtained from Endocrine Sciences (Tarzana, CA) (cortisol and androstenedione) and Radioassay System Laboratories (Carson, CA) (corticosterone and aldosterone). cAMP was measured using antisera purchased from Miles–Yeda Laboratories (Rehovot, Israel), and radioiodinated cAMP was from New England Nuclear.

Peptides and Protein Preparation. Rat ACTH-(1–39) was synthesized by solid-phase methodology in this laboratory (13). Basic FGF was purified to homogeneity from bovine pituitary glands in this laboratory (1), and highly purified TGF-β from human platelets was generously supplied by M. B. Sporn (National Cancer Institute, Bethesda, MD) (14).

Cell Number Determination. Bovine adrenocortical cells were cultured as described and treated with 1 ml of a trypsin/EDTA solution containing 0.9% NaCl, 0.01 M sodium phosphate, 0.05% trypsin, and 0.02% EDTA (pH 7.4). The cells were incubated at 37°C, and cell number was determined with a Coulter particle counter.

Statistical Analysis. Comparisons of the results obtained between the various treatments were conducted by the multiple comparison test of Dunnett.

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Abbreviations: TGF-β, transforming growth factor type β; FGF, fibroblast growth factor; LDL, low density lipoprotein; ACTH, corticotropin.

RESULTS

Differential Effect of TGF-β on Adrenocortical Growth and Function. The first test of TGF-β activity on adrenocortical cells was designed to investigate the possibility that, like FGF, it might modulate cell growth. As expected, the incubation of the bovine adrenocortical cells with basic FGF resulted in a significant increase in cell number (Fig. 1). In contrast, the growth of the adrenocortical cells was not modified by TGF-β. Moreover, the addition of TGF-β did not modify the mitogenic response to FGF. In similar experiments with ACTH, well known for its anti-mitotic activity *in vitro* (7, 15), there were also no interactions with TGF-β (Table 1): incubations with ACTH significantly reduced cell growth; addition of TGF-β at 100 pg/ml had no effect on the growth inhibiting response to ACTH.

The effects of TGF-β on steroidogenesis are shown in Fig. 2. At concentrations as low as 4×10^{-13} M TGF-β (10 pg/ml) significantly inhibits cortisol formation. The inhibition was dose dependent and maximal inhibition was detected with 4×10^{-11} M TGF-β (1 ng/ml). In the presence of ACTH (Fig. 2*B*), TGF-β was just as effective in inhibiting the formation of cortisol as when basal (Fig. 2*A*) steroidogenesis was examined. In each instance, cortisol formation was reduced by 50–60% but was never completely inhibited. TGF-β also reduced the steroidogenic response in terms of aldosterone, corticosterone, and Δ^4-androstenedione formation (data not shown) suggesting that the inhibition of steroid formation occurs at sites prior to their common precursor, progesterone. This hypothesis is clearly supported by the observation that the addition of 25-hydroxycholesterol, pregnenolone, or progesterone to the cells completely reverses the inhibitory effects of TGF-β (Fig. 3). The fact that low density lipoprotein (LDL) could not restore steroidogenesis to control levels indicates that the effect of TGF-β occurs at the level of the conversion of cholesterol esters to cholesterol (i.e., cholesterol ester hydrolase).

The addition of TGF-β to the cells resulted in a shift in the maximal response to ACTH (Fig. 4). In contrast, the ED_{50} of the dose–response curves to ACTH remained in the range of $2–3 \times 10^{-11}$ M. This result is consistent with a noncompetitive interaction between TGF-β and ACTH and suggests that both molecules are acting on different sites in the adrenocortical cell. They also support the hypothesis that TGF-β acts at sites distal to the ACTH receptor, most likely at the enzymes that catalyze steroidogenesis.

The Course of the Effect of TGF-β. The noncompetitive interaction between ACTH and TGF-β leads to the hypothesis that a second messenger is interrupting the steroidogenic response. An examination of the time course of the effects of TGF-β supported this notion and revealed that a minimum of 16–20 hr were required prior to detecting a change in the formation of steroids (Fig. 5*A*). This result is in contrast to those obtained with ACTH, which acts with a far shorter lag phase (16). A minimum of 12–18 hr was required before cells treated with TGF-β showed a steroidogenic response that was similar to untreated cells (Fig. 5*B*).

Table 1. Effect of TGF-β and ACTH on the growth of bovine adrenocortical cells

Treatment	Cells, no. × 10^{-4}*
Control	1.70 ± 0.10
TGF-β (10 pg/ml)	1.99 ± 0.07
TGF-β (100 pg/ml)	1.84 ± 0.07
TGF-β (1 ng/ml)	1.71 ± 0.08
1 nM ACTH	1.04 ± 0.04†
1 nM ACTH/TGF-β (10 pg/ml)	0.91 ± 0.06†
1 nM ACTH/TGF-β (100 pg/ml)	1.04 ± 0.08†
1 nM ACTH/TGF-β (1 ng/ml)	1.07 ± 0.07†

*Cell counts were obtained on the fourth day of culture.
†$P < 0.05$ compared to control cells. All other differences were not significant.

Specificity of the Effect of TGF-β. As shown in Table 2, TGF-β at a concentration of 1 ng/ml had no effect on the formation of cAMP. In these experiments, although TGF-β was effective in reducing the steroidogenic response, no differences in the formation of cAMP were observed when adenylate cyclase was stimulated by ACTH and forskolin. Similar results were obtained when phosphodiesterase activity was inhibited by isobutylmethylxanthine.

DISCUSSION

The results presented here demonstrate a potent inhibitory effect of TGF-β on adrenocortical steroidogenesis. The following evidence suggests that the effects of TGF-β are not mediated by cell toxicity: cell growth is unaffected by the presence of TGF-β whether in control or FGF-treated cells, and cell viability at all times could be monitored by trypan blue exclusion. Moreover, a visual inspection of control and treated cells failed to show the presence of cellular debris.

We have investigated what specific step of steroidogenesis is inhibited by TGF-β. While ACTH activates adenylate cyclase and the formation of cAMP results in increased steroid hormone synthesis (17), this process is not inhibited by TGF-β. Vilgrain *et al.* (18) have reported that the

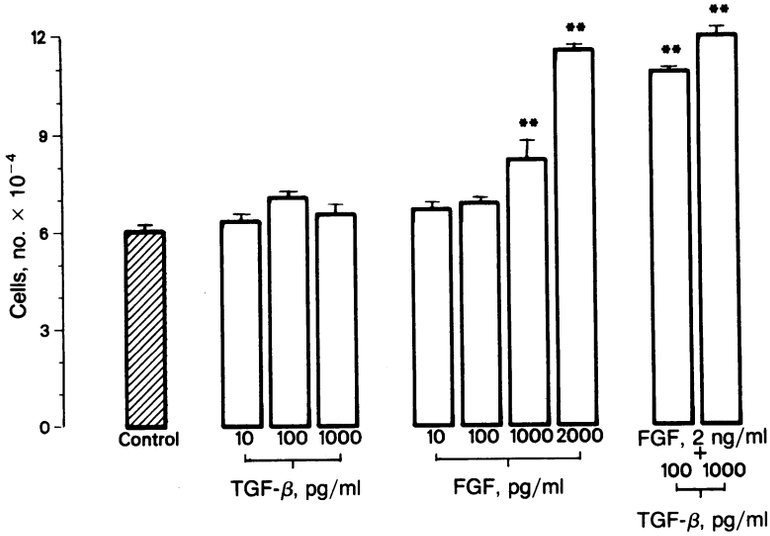

FIG. 1. Effect of TGF-β on bovine adrenocortical cell growth. TGF-β or FGF was added in 10-μl aliquots every 48 hr to bovine adrenocortical cells in culture at the concentrations indicated. On the seventh day, cells were trypsinized and counted on a Coulter particle counter. Each bar represents mean ± SEM of six samples. ** indicates a significant increase of cell number compared with control ($P < 0.05$).

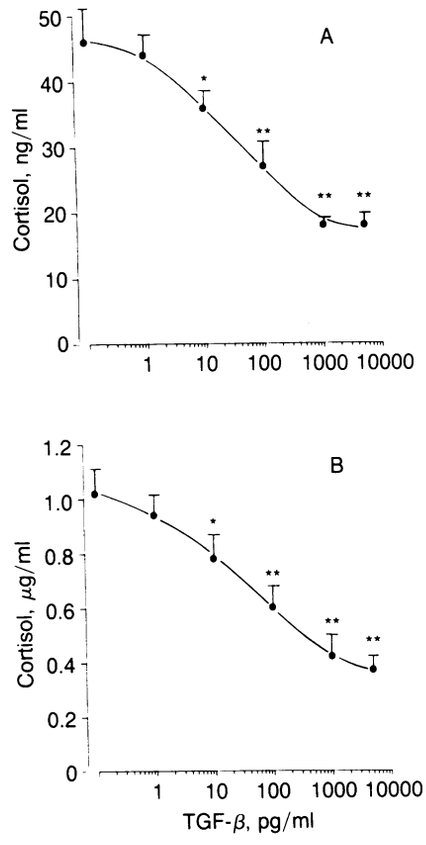

FIG. 2. Effect of TGF-β on cortisol formation. TGF-β was added to control (*A*) or ACTH (1 nM)-stimulated (*B*) adrenocortical cells for 24 hr. The incubation medium was collected, and the formation of cortisol was measured by RIA. The results are the mean ± SEM of four samples. (* $P < 0.1$ and ** $P < 0.05$.)

steroidogenic effects of ACTH can also be mediated by protein kinase C. The effects of TGF-β on this enzyme in the adrenal are not known. It is, however, a key step in the signal transduction of several growth factors as well as phorbol esters (19). Since TGF-β suppresses all three of the steroidogenic pathways derived from progesterone (including cortisol, corticosterone, aldosterone, and Δ^4-androstenedione), it is likely that TGF-β alters steroidogenic enzyme activities prior to the formation of this steroid. These steps, which involve regulation of 3β-hydroxysteroid dehydrogenase, cholesterol side chain cleavage, and the hydrolysis of stored and exogenous cholesterol esters are highly sensitive to several hormonal effectors including ACTH, γ-melanocyte-stimulating hormone, and estrogens (3, 20, 21). It is also important to consider the possibility that TGF-β may also regulate the cellular uptake of lipoprotein precursors. The ability of exogenous progesterone, pregnenolone, and 25-hydroxycholesterol, but not LDL, to reverse the inhibitory effect of TGF-β demonstrates that it is the availability of free cholesterol that is rate limited by the growth factor. In the bovine adrenocortical cell system, *de novo* cholesterol synthesis is extremely low, and the cells are almost completely dependent on an exogenous supply of cholesterol (22). Cholesterol ester hydrolase catalyzes the hydrolysis of both stored cholesterol esters and those provided from LDL. ACTH and cAMP stimulate this process, and factors like γ-melanocyte-stimulating hormone can increase its activity and potentiate the effects of ACTH through a mechanism independent of cAMP (4, 23). The present study suggests that TGF-β interferes with this same process again through a cAMP independent pathway.

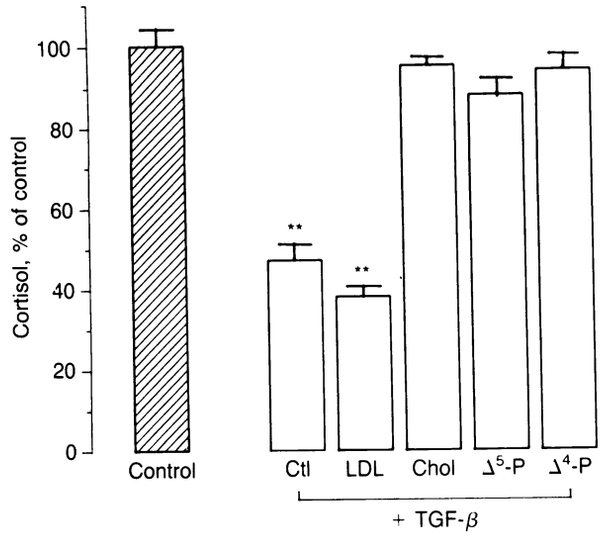

FIG. 3. Effect of progesterone, pregnenolone 25-hydroxycholesterol, and LDL on cortisol formation. Adrenocortical cells were treated with TGF-β (1 ng/ml) in the presence of no exogenous precursors (Ctl), LDL at 20 μg/ml, 25-hydroxycholesterol (Chol, 25 μg/ml), pregnenolone (Δ^5-P, 10 μM), or progesterone (Δ^4-P, 10 μM) in serum-free medium for 24 hr. The hatched bar represents control. The incubation medium was collected, and the formation of cortisol was measured by RIA. The results are the mean of eight replicate samples and were normalized to the mean of their respective controls (i.e., no TGF-β treatment). In each instance the addition of exogenous LDL, 25-hydroxycholesterol, pregnenolone, or progesterone was capable of increasing basal cortisol formation indicating that the addition of precursor provoked increased steroid synthesis. (**, $P < 0.05$.)

The 16–20 hr of lag time required for the detection of inhibition suggest that there is a possible induction of a second messenger that mediates the effects of TGF-β. There is also a second possibility, however, to explain the lag phase required to observe the TGF-β-mediated inhibition of steroidogenesis. The observation that the effects of TGF-β cannot be reversed by LDL but can be reversed by steroid precursors would predict that the inhibition of steroidogenesis would only be detected after the exhaustion of the

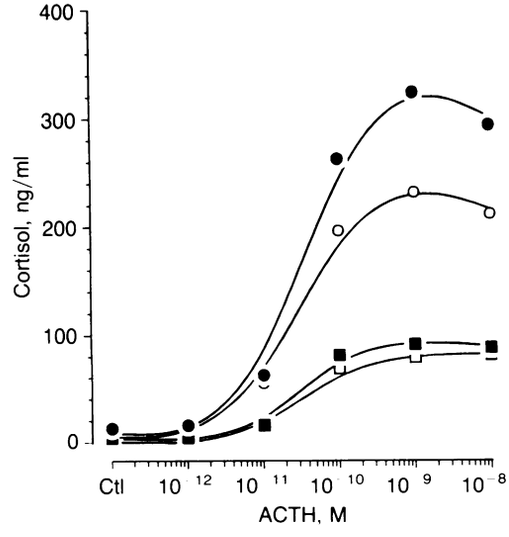

FIG. 4. Noncompetitive interaction between TGF-β and ACTH. Adrenocortical cells were treated with 10^{-12} to 10^{-8} M ACTH alone (●) or in the presence of TGF-β at 10 pg/ml (○), at 100 pg/ml (■) and at 1 ng/ml (□) for 24 hr. The incubation medium was collected and the formation of cortisol was measured by RIA.

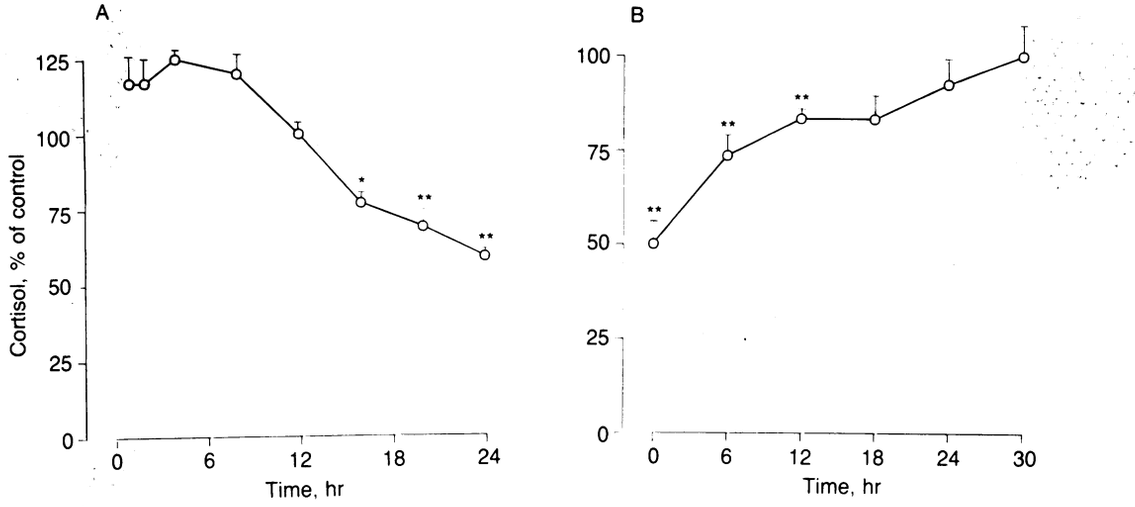

FIG. 5. Time course of the effect of TGF-β. (*A*) Bovine adrenocortical cells were treated with TGF-β at 100 pg/ml for the times indicated, and the formation of cortisol was measured by RIA. The results were normalized to the control value obtained at each time point and are expressed as the mean ± SEM of four samples. (*B*) Cells were treated with TGF-β for 24 hr, washed twice, and incubated with 1 nM ACTH for 24 hr at 0–30 hr after the treatment with TGF-β. The formation of cortisol was measured by RIA at each of the time points indicated and was normalized to its appropriate control. (* $P < 0.1$ and ** $P < 0.05$.)

intracellular pools of free cholesterol. Because both basal and ACTH-stimulated steroidogenesis are modified by TGF-β, it seems likely that the enzymes involved in the steroidogenic pathways are continuously affected by the growth factor regardless of a concomitant stimulation of adenylate cyclase. This observation is consistent with the noncompetitive nature of the interaction between ACTH and TGF-β and is compatible with the hypothesis that the effects of TGF-β are at least partially mediated by a depletion in free intercellular cholesterol pools.

It is interesting to note that, as in the case of the effects of FGF on pituitary (12) and ovarian (24) functions, TGF-β has an effect on adrenocortical cells that can be totally dissociated from any modification of cell growth. This result has now been widely observed (12, 24–27) and suggests that the physiological functions of many, if not all, growth promoting and growth inhibiting substances may have no correlation with activities for which they were first described. Thus TGF-β, originally isolated and characterized on the basis of its ability to induce anchorage-independent cell growth (i.e., its transforming activity), has been found to have a wide range of diverse activities on many cell types (28–34). *In vivo* studies and the availability of antisera and large quantities of growth factor should enable an assessment of its role in maintaining normal cellular homeostasis in the adrenal.

The effect of TGF-β on the inhibition of steroidogenesis in the adrenal is unlike the effect of atrial natriuretic factor in many ways. First, atrial natriuretic factor inhibits the formation of aldosterone in acute studies requiring less than a 2-hr incubation *in vitro* (5, 35–37). Second, it does not modify the steroidogenic response distal to cAMP formation. Its mechanism of action is associated with its ability to increase the formation of cGMP (37). TGF-β on the other hand requires significantly longer incubation times and appears to act specifically on cholesterol formation. The net result is an across the board decrease in the formation of Δ^4-steroid products.

While the exact physiological significance of the findings reported here remain to be established, Baird *et al.* (38) have proposed a role for corticostatic factors that acts locally to modulate adrenocortical function. These factors, peptidic in nature and partially purified from adrenomedullary tissue extracts, were examined because of the wide number of clinical and experimental studies that are associated with idiopathic modifications in adrenocortical function. These include the adrenocortical responses that occur during hemorrhage (39) as well as the modified patterns of steroid formation observed during fetal development (1, 40) and adrenarche (2). As one particular example, severe Gram-negative bacterial infections, such as those that occur in endotoxemia, are often associated with adrenocortical insufficiency (9). While endotoxins themselves have no effect on

Table 2. Effect of TGF-β on cAMP formation

Treatment	Cortisol,* μg/ml	cAMP	
		Extracellular, pmol/ml	Intracellular, pmol per 10^4 cells
Control	0.29 ± 0.01	<1.0	<1.0
1 nM ACTH/TGF-β (1 ng/ml)	3.11 ± 0.09	119.3 ± 8.7	93.0 ± 6.5
	1.58 ± 0.01†	135.5 ± 13.7	112.0 ± 14.0
10 μM Forskolin/TGF-β (1 ng/ml)	3.31 ± 0.14	132.5 ± 13.0	115.5 ± 5.5
	1.87 ± 0.05†	129.1 ± 11.3	106.0 ± 6.0
0.1 mM iBtMeXan/TGF-β (1 ng/ml)	0.86 ± 0.02	10.2 ± 0.9	35.0 ± 2.5
	0.21 ± 0.01†	9.4 ± 0.9	43.5 ± 6.0

iBtMeXan, isobutylmethylxanthine.
*Measurements were made from 24-hr incubations with each treatment.
†$P < 0.05$ compared to the results from cells that did not receive TGF-β. All other differences were not significant. Results are the mean of six determinations.

reducing steroidogenesis (11), supernatants obtained from lipopolysaccharide-stimulated macrophages significantly modify adrenocortical function (41). The activity in this instance has all of the characteristics described here for TGF-β. It suppressed ACTH-induced steroidogenesis, required an 18-hr incubation, and had no effect on cell growth. Like TGF-β, the pattern of the response suggested a disruption of steroidogenesis at steps distal to the formation of cAMP. Because of these results and the demonstration that monocytic cell types are an integral part of the adrenal cortex (42, 43), it may be necessary to consider the importance of monocyte-derived products in the physiological and pathophysiological regulation of the stress response.

We are grateful to Dr. Nicholas Ling for providing the synthetic rat ACTH used in these studies; to Drs. Michael Sporn and Richard Assoian for their generous gift of TGF-β; to Terri Durkin, Carolyn Wong, Robert Klepper, and Vivianne Bonora for excellent technical support; and to Denise Higgins and Darleen Gore for assistance in the preparation of the manuscript. This research was supported by program grants from the National Institutes of Health (AM-18811 and HD-09690) and by the Robert J. and Helen C. Kleberg Foundation.

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