Transforming growth factor β1 inhibits aldosterone and cortisol production in the human adrenocortical cell line NCI-H295R through inhibition of CYP11B1 and CYP11B2 expression

P Liakos, D Lenz1, R Bernhardt2, J-J Feige and G Defaye

INSERM EMI 01–05, Department of Cell Regulation and Dynamics, Commissariat à l’Energie Atomique/Grenoble, 17 Rue des Martyrs, F-38054 Grenoble Cedex 9, France
1Max-Delbrück-Centrum für Molekulare Medizin, Berlin-Buch, Germany
2Universität des Saarlandes, FR Biochemie, Saarbrücken, Germany

(Requests for offprints should be addressed to J-J Feige; Email: jjfeige@cea.fr)
(Correspondence should be addressed to G Defaye; Email: gdefaye@cea.fr)
(P Liakos is now at Department of Biochemistry, Medical School, Aristotle University of Thessaloniki, 54210 Thessaloniki, Greece)

Abstract

Transforming growth factor β1 (TGFβ1) has been shown to exert strong inhibitory effects on adrenocortical cell steroidogenesis. However, the molecular targets of TGFβ1 in adrenocortical cells appear to differ between species. Here, we report the first characterization of the regulatory effects of TGFβ1 on the steroidogenic functions of the human adrenocortical tumor cell line NCI-H295R. After treatment with 2 ng/ml TGFβ1 for 24 h, basal production of corticosterone, cortisol and androstenedione was dramatically decreased. When TGFβ1 was added simultaneously with forskolin, the production of cortisol and 11-hydroxyandrostenedione was decreased by 85% whereas that of deoxycorticisol was increased. When TGFβ1 was added simultaneously with angiotensin II, aldosterone production was reduced by 80%. We observed that TGFβ1 strongly inhibits forskolin-induced steroid 11β-hydroxylase activity and CYP11B1 mRNA levels, as well as angiotensin II-induced aldosterone synthase activity and CYP11B2 mRNA levels. CYP11B1 and CYP11B2 gene products thus appear as the major steroidogenic enzymes down-regulated by TGFβ1 in the human adrenocortical tumor cell line NCI-H295R.


Introduction

There is increasing evidence that the biological functions of growth factors extend far beyond their mitogenic activity. In this way, transforming growth factor β1 (TGFβ1) which was initially characterized as an inducer of anchorage-independent cell growth is now recognized as a multifunctional regulator of an array of biological processes including cell differentiation, cell migration, extracellular matrix formation, immunosuppression, angiogenesis, and steroidogenesis. Since the initial reports published in 1986 (Feige et al. 1986, Hotta & Baird 1986), the regulatory effects of TGFβ1 on adrenocortical cell steroidogenic functions have been characterized in some detail in primary cultures of glomerulosa and fasciculata cells from various species. Although TGFβ1 appears as a strong inhibitor of steroidogenesis in each of these models, the nature of the primary targets down-regulated by this factor greatly differs from one cell type to another and as a function of the metabolic status of a given cell type. In freshly prepared primary cultures of bovine adrenocortical fasciculata cells, we observed that both basal and adrenocorticotropin (ACTH)-induced cortisol production was inhibited by TGFβ1 through the down-regulation of steroidogenic acute regulatory protein (StAR) and steroid 17α-hydroxylase (the product of CYP17) expression (Feige et al. 1987, Perrin et al. 1991, Brand et al. 1998b). However, the relative extent of inhibition of these two genes appeared to vary as a function of the age of the primary culture, with CYP17 being less prominently inhibited on day 1 than on day 4 of primary culture in the presence of ACTH (Brand et al. 1998a). Interestingly, using primary cultures of bovine fasciculata cells that were frozen on the day of preparation and thawed before experiments, Hotta and Baird (1987) observed that low density lipoprotein (LDL) uptake via the LDL receptor was the primary target of TGFβ1 action. Using freshly prepared bovine fasciculata cells that did not undergo a freeze/thaw cycle, we observed a similar effect of TGFβ1 on LDL receptors but, since LDL uptake was not limiting for steroidogenesis in these cultures, it did not appear to significantly contribute to the inhibition of cortisol
production (Feige et al. 1991b). These discrepancies suggest that, depending on the cellular metabolic requirements, different steps of the cortisol biosynthesis pathway are involved in the negative regulation of steroidogenesis by TGFβ1. In fasciculata cells from other species, other combinations of primary targets have been identified: cholesterol supply, steroid 17α-hydroxylase and ACTH receptors in ovine cells (Rainey et al. 1988, 1989, 1990), and inhibition of dehydroepiandrosterone sulfate synthesis in the absence of any effect on cortisol synthesis in adult human cells (Lebrethon et al. 1994). Interestingly, TGFβ1 was found to inhibit dehydroepiandrosterone sulfate and, to a lesser extent, cortisol production by both fetal zone and neocortex cells of the human fetal adrenal (Stankovic et al. 1994). Inhibition of both steroid 17α-hydroxylase and steroid sulfotransferase expression was observed in these cells (Parker et al. 1998).

The aim of the present work was to characterize the effects of TGFβ1 on the steroidogenic functions of the human adrenocortical tumor cell line NCI-H295R and to identify its main molecular targets. The NCI-H295 cell line was first established from a human adrenocortical carcinoma and maintained in culture for 10 years by Gazdar et al. (1990) before being released to the scientific community. A subclone presenting improved adherence to plastic was further isolated by Rainey et al. (1993, 1994) and named NCI-H295R. Both the original cell line and the adherent subclone have the same peculiar steroid secretion profile that also appears to be preserved after subcutaneous xenografting into nude mice: these tumor cells secrete all three classes of corticosteroids, namely mineralocorticoids, glucocorticoids and androgens, and express the appropriate steroidogenic enzymes (Bird et al. 1993, Rainey et al. 1993, Staels et al. 1993, Logié et al. 2000). After stimulation by forskolin, 11β-hydroxyandrostenedione is the major C-19 steroid secreted by NCI-H295R cells (Rainey et al. 1993); this is also true for bovine fasciculata cells (Chabre et al. 1993). 11β-Hydroxyandrostenedione, which has weak androgenic activity but stimulates bone formation, is produced by two biosynthetic pathways in humans: 11β hydroxylation of androstenedione in the adrenocortical zona reticularis and side chain cleavage of cortisol at the C-17 position in the liver (Suzuki et al. 2000). Although its secretion profile is uncommonly observed in spontaneous human adrenal carcinomas (Logié et al. 1999).

The aim of this study was to characterize the effects of TGFβ1 on basal, ACTH-stimulated and angiotensin II-stimulated corticosteroid production by NCI-H295R. For this, we combined steroid profile analyses, enzymatic assays and gene expression studies.

Materials and Methods

Reagents

All chemicals were purchased either from Roche Diagnostics (Meylan, France) or from Sigma (St Louis, MO, USA). Silica gel plates 60F254 were from Merck (Darmstadt, Germany). Synthetic ACTH(1–24) (Synacthen) was provided by Novartis (Basel, Switzerland). [1,2,6,7-3H]Dehydroepiandrosterone (64·5 Ci/mmol), [7-3H-(N)]pregnenolone (25 Ci/mmol), [1,2,6,7-3H]aldosterone (70 Ci/mmol) and [1,2,3,4-3H]11-deoxycortic (41·7 Ci/mmol) were from NEN Life Science Products (Zaventem, Belgium). [1,2,6,7,8-3H]Corticosterone (60 Ci/mmol), [1,2,6,7-3H]cortisol (100 Ci/mmol) and [1,2,6,7,17-3H]17-hydroxyprogesterone (89 Ci/mmol) were from Amersham Pharmacia Biotech (Orsay, France).

Steroid determinations

Pregnenolone, cortisol and aldosterone were measured in cell culture medium using specific radioimmunoassays. Cortisol was measured directly in medium whereas pregnenolone and aldosterone were extracted first in dichloromethane. The anti-cortisol antiserum was from Endocrine Sciences (Calabasas Hills, CA, USA), the anti-pregnenolone antiserum was from Biogenesis (Poole, Dorset, UK) and the anti-aldosterone antiserum was a generous gift from Dr B Aupetit (Hôpital Pitié-Salpetriere, Paris, France).

Cell culture

The human NCI-H295R adrenocortical cancer cell line (Rainey et al. 1994) was kindly provided by Dr W Rainey (University of Texas Southwestern Medical Center, Dallas, TX, USA). The cells were maintained in Dulbecco’s modified Eagle’s–Ham’s F-12 medium (DMEM/F12, 1/1, v/v) supplemented with 1% ITS+ (insulin 1 µg/ml, transferrin 1 µg/ml, selenium 1 ng/ml, linoleic acid 1 µg/ml, final concentrations), 2% Ultroser SF and antibiotics. ITS+ and Ultroser SF were respectively from Collaborative Biomedical Products (Bedford, MA, USA) and BioSepra SA (Villeneuve la Garenne, France).

HPLC analysis of steroid profiles

Conditioned medium was supplemented with a known amount of 21-acetyldeoxycortisol (used as an internal
standard) and was extracted with 2 vol. dichloromethane. The organic phase was taken to dryness under a nitrogen stream and the residue was dissolved in 0·05 ml methanol for injection into the HPLC system (Gold, Beckman). Steroid separation was performed on a Lichrospher (5 µm, Beckman) C18 column (isoelectric 4 mm, height 25 cm) with a methanol–water gradient (50–75%) at a flow rate of 0·5 ml/min. Individual steroids were detected by their absorbance at 240 nm and identified by comparing their retention times to those of authentic samples. They were quantitated by reference to the known concentration of the internal standard.

Cell lysis and protein determination
After medium removal, the cells were lysed in sodium phosphate buffer (10 mM, pH 7·4) containing 150 mM NaCl, 0·1% SDS, 1% deoxycholate, 1% Triton X-100 and 0·2 mM phenylmethylsulfonylfluoride. Protein concentration of the extracts was then determined using the bicinchoninic protein assay kit (MicroBCA Protein Assay kit from Pierce, Rockford, IL, USA).

Individual enzymatic activities
Cholesterol side chain cleavage activity was measured by incubating the cells for 2 h at 37 °C with 25-hydroxycholesterol (25 µM) in the presence of trilostane (2 µM) (a generous gift from Winthrop Laboratories, France) and SU 10603 (5 µM) (Novartis), two inhibitors of 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17-hydroxylase activities respectively. After extraction of the steroids in dichloromethane, pregnenolone was quantitated by RIA.

Steroid 17α-hydroxylase activity was assayed by measuring the transformation of [3H]pregnenolone into [3H]17α-hydroxypregnenolone. The cells were incubated for 2 h at 37 °C in Ham’s F12 medium containing 50 µM [3H]pregnenolone and 2 µM trilostane. The steroids were extracted from the medium in chloroform. The organic extract was analyzed by thin layer chromatography on silica gel plates (F-254, Merck) in chloroform:ethyl acetate (1:1, v/v) and their radioactive content was measured as described above.

Steroid 11β-hydroxylase activity was measured using [3H]deoxycorticosterone (100 µM) as a substrate. [3H]Cortisol and [3H]deoxycorticosterol were separated by thin-layer chromatography in chloroform:ethyl acetate (1:1, v/v) and their radioactive content was measured as described above.

Aldosterone synthase activity was measured after incubation of the cells with corticosterone (10 µM) for 2 h. Produced aldosterone was measured by radioimmunoassay.

Ribonuclease protection assay
cDNA probes for P450aldo, P450c11 and actin (cloned in pCRII) were generous gifts from Dr W E Rainey and Dr W Freige (University of Texas, Dallas, TX, USA) (Holland et al. 1993, Denner et al. 1996).

The linearized plasmids were used in a transcription reaction with [α-32P]CTP and T7 RNA polymerase using the MAXiscript in vitro transcription kit (Ambion, Austin, TX, USA). RNase protection assay was performed using the RPA II Ribonuclease Protection Assay kit (Ambion). Protected RNA species were resolved by electrophoresis on a denaturing acrylamide (5%)-urea (8 M) gel. pUC19 was digested with Sau 3A1 then end-labeled with [32P]dCTP and 1 U Klenow DNA polymerase. The [32P]labeled DNA fragments were migrated in a parallel lane as molecular size standards.

Radiolabeled bands were visualized by scanning with a β-imager (PhosphorImager, Molecular Dynamics, Sunnyvale, CA, USA) and quantitated using the Image Quant software (Molecular Dynamics).

DNA transfection and dual luciferase assay
The plasmids used contain several deletion constructs of the 1993 bp-long hCYP11B1 promoter fused to the luciferase gene in the pGL3 vector.

NCI-H295R cells were transfected by electroporation (250 mV, 450 µF) using a gene pulsor apparatus (Eurogentec, Angers, France). Control pRL-TK plasmid (1·2 µg; Promega Corp.) and reporter luciferase constructs (4·8 µg) were cotransfected into 3 × 106 cells and the cells were subsequently plated into a 12-well culture plate. After 24 h of culture, the transfected cells were treated for an additional 24-h period without or with TGFβ1 (2 ng/ml), forskolin (10 µM) or both. The cells were then harvested, lysed and both firefly and renilla luciferase
activities were sequentially measured with the Dual-luciferase reporter assay system (Promega Corp.) on a LUMAT LB 9507 luminometer (EGG Berthold, Bad Wildbad, Germany). Results were expressed as relative firefly luciferase light units (RLU) normalized to renilla luciferase activity of the same sample.

Statistical analysis
Statistical analysis of the data was performed by analysis of variance followed by Student’s t-test. Values are considered significant when P<0·05.

Results

Steroid production by NCI-H295R cells is inhibited by TGFβ1

UV-detectable steroids secreted by NCI-H295R cells over a period of 4 h were separated by HPLC and quantitated. As shown in Fig. 1A, corticosterone is the major steroid secreted under basal conditions, but cortisol and androstenedione are also secreted in lesser quantities. When cells were treated with 2 ng/ml TGFβ1 for 48 h and the production of steroids secreted over a period of 4 h was subsequently measured, we observed a dramatic decrease in the production of corticosterone, cortisol and androstenedione (Fig. 1A). Under forskolin treatment, cortisol became the major secreted steroid but 11β-hydroxysteriodenedione and corticosterone were also abundant. Androstenedione and deoxycorticisol were also detectable (Fig. 1A). Simultaneous incubation with TGFβ1 and forskolin decreased the production of both cortisol and 11-hydroxysteriodenedione by 85% as compared with forskolin-treated cells and increased that of deoxycorticisol (Fig. 1A). The ratio of deoxycorticisol to cortisol was shifted from 0·1 in forskolin-treated cells to about 2·5 in TGFβ1-treated cells, suggesting that TGFβ1 inhibits steroid 11β-hydroxylation, the very last step in cortisol biosynthesis. To confirm this hypothesis, NCI-H295R cells were treated with forskolin in the absence or presence of TGFβ1 and subsequently supplied with high concentrations of 17-hydroxyprogesterone (20 µM). The production of steroid end-products was then measured. In agreement with our hypothesis, cortisol production decreased whereas deoxycorticisol production increased after TGFβ1 treatment (Fig. 1B). In addition, a moderate inhibition of androstenedione production was observed, suggesting that steroid 17α-hydroxylaše might also be inhibited by TGFβ1.

We then studied the time-course of the inhibition of cortisol production by TGFβ1. Cells were incubated with 2 ng/ml TGFβ1 for various periods of time (from 2 to 48 h) and the amount of cortisol secreted over a window period of 2 h following medium renewal was then measured. As shown in Fig. 2, basal production of cortisol was not significantly modified by TGFβ1 for up to 24 h and was reduced by 70% after 48 h. Under forskolin treatment, the production of cortisol was gradually stimulated between 7 and 36 h and reached a maximal sevenfold increase after 36 h and 48 h. When TGFβ1 was added together with forskolin, the increase in cortisol production was strongly reduced. TGFβ1 appeared to inhibit forskolin-induced cortisol production by 88% after 48 h and this effect was both time-dependent (Fig. 2) and dose-dependent (EC_{50}=50 pg/ml TGFβ1; data not shown).

In order to check whether additional steps of the steroidogenic pathway upstream of 11β-hydroxylation were also affected by TGFβ1, we measured the conversion of exogenously added steroid precursors such as 25-hydroxycholesterol (25-OH cholesterol), a cell permeant analog of cholesterol, 17α-hydroxypregnenolone (17-OH pregnenolone) or 17α-hydroxyprogesterone (17-α-progesterone) into cortisol. NCI-H295R cells were treated for 48 h with forskolin in the presence or absence of TGFβ1; they were subsequently washed, incubated for 2 h in the presence of the steroid substrates and cortisol was then measured in the medium. As shown in Table 1, in the absence of exogenous substrate, TGFβ1 inhibited forskolin-induced cortisol production by 90%. This inhibition was not significantly modified when 25-hydroxycholesterol or pregnenolone were given to the cells (85% inhibition) and was only slightly reduced to 79% and 71% respectively when 17α-hydroxypregnenolone or 17α-hydroxyprogesterone were supplied as substrates. To determine more precisely the effect of TGFβ1 on the successive steps of the steroidogenic pathway, we measured each enzymatic activity separately (Table 2). Under basal conditions, the activities of cholesterol side chain cleavage, 3β-HSD, 17α-hydroxylase, 21-hydroxylase and 11β-hydroxylase were not significantly modified by TGFβ1 treatment. In the presence of forskolin, TGFβ1 inhibited the conversion of cholesterol into pregnenolone by 8% whereas it reduced the conversion of 25-hydroxycholesterol into pregnenolone by only 6% (see cholesterol side chain cleavage activity determinations in Table 2). This was in agreement with the mild inhibitory effect of TGFβ1 on StAR expression that we previously characterized in NCI-H295R cells (Brand et al. 1998c). TGFβ1 treatment also inhibited to various extents the forskolin-induced levels of several other enzymes of the pathway: 3β-HSD by 19%, 17α-hydroxylase by 29%, 21-hydroxylase by 8% and 11β-hydroxylase by 79%, confirming that this latter enzymatic step was the main target of TGFβ1 action in NCI-H295R cells.

The time-course of the effect of TGFβ1 on 11β-hydroxylase activity was then studied in more detail. As shown in Fig. 3, continuous treatment of NCI-H295R cells with TGFβ1 (2 ng/ml) had no effect on the basal
Figure 1 HPLC analysis of the steroid secretion profiles of NCI-H295R cells under various culture conditions. (A) NCI-H295R cells were treated for 48 h in the absence (control) or presence of TGFβ1 (2 ng/ml), forskolin (10 μM), or forskolin + TGFβ1. The medium was then replaced with fresh medium and the steroids secreted during a further 4-h incubation period were extracted and separated by HPLC. The amount of secreted steroids was quantitated from the HPLC profiles by reference to an internal standard (21-acetyldeoxycortisol) added to the medium before extraction. After collection of the medium, cells were solubilized in lysis buffer and analyzed for protein content. The amount of secreted steroids was normalized to the amount of cellular proteins. Each value represents the mean ± S.E.M. of sample analyses from two independent experiments. 11-OH AD, 11-hydroxyandrostenedione. (B) NCI-H295R cells were treated for 48 h in the presence of forskolin or both forskolin and TGFβ1. Fresh medium containing 20 μM 17-hydroxyprogesterone was then added. Steroids secreted during a further 4-h incubation period were analyzed as in (A). Each value represents the mean ± S.E.M. of sample analyses from two independent experiments.
activity of 11β-hydroxylase, but inhibited forskolin-induced 11β-hydroxylase activity with a kinetics that was slightly slower than the stimulating effect of forskolin. Whereas the initial stimulating effect of forskolin was observed after 12 h of treatment, the initial inhibitory effect of TGFβ1 was observed after 18 h. The maximal effects of both forskolin and TGFβ1 reached a plateau between 36 and 48 h.

Table 1 TGFβ1 inhibition of the conversion of steroid precursors into cortisol. Results are expressed as the cortisol production (nmol/h/mg protein) and are the means ± S.E.M. of triplicate sample determinations. This experiment is representative of three independent experiments.

<table>
<thead>
<tr>
<th>Precursor added</th>
<th>Cortisol production (nmol/h/mg protein)</th>
<th>Mean % inhibitionb(n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>TGFβ1</td>
</tr>
<tr>
<td>None</td>
<td>0·45 ± 0·04</td>
<td>0·055 ± 0·007</td>
</tr>
<tr>
<td>25-OH cholesterol (50 μM)</td>
<td>1·23 ± 0·05</td>
<td>0·05 ± 0·10</td>
</tr>
<tr>
<td>Pregnenolone (20 μM)</td>
<td>1·43 ± 0·2</td>
<td>0·21 ± 0·02</td>
</tr>
<tr>
<td>17-OH pregnenolone (20 μM)</td>
<td>9·3 ± 0·7</td>
<td>1·9 ± 0·3</td>
</tr>
<tr>
<td>17-OH progesterone (20 μM)</td>
<td>14·9 ± 1·2</td>
<td>3·4 ± 0·4</td>
</tr>
</tbody>
</table>

NCI-H295R cells were incubated for 48 h with 10 μM forskolin in the presence or in the absence of 2 ng/ml TGFβ1. The medium was then renewed and the cells were incubated for 2 h in fresh medium containing the indicated steroid substrates. Production of cortisol in the medium was measured by RIA and normalized to the cellular protein content.

*Percentage inhibition of cortisol production observed in TGFβ1-treated cells compared with untreated cells in this experiment. bMean percentage inhibition ± S.E.M. of cortisol production observed in TGFβ1-treated cells compared with untreated cells as determined from three independent experiments.
Table 2 TGFβ1 inhibition of the successive enzymatic steps of the cortisol biosynthesis pathway. The results are expressed as the mean ± S.E.M. of triplicate sample determinations. This experiment is representative of three independent experiments.

<table>
<thead>
<tr>
<th>Activity (nmol/h/mg protein)</th>
<th>Inhibition (%)</th>
<th>Mean % inhibition (n=3)</th>
<th>Activity (nmol/h/mg protein)</th>
<th>Inhibition (%)</th>
<th>Mean % inhibition (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td>Forskolin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol side chain cleavage</td>
<td>5</td>
<td>7 ± 2</td>
<td>7 ± 2</td>
<td>14</td>
<td>8 ± 5</td>
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<tr>
<td>Cholesterol, 48 h</td>
<td>0.036 ± 0.007</td>
<td>0.034 ± 0.004</td>
<td>0.043 ± 0.003</td>
<td>0.037 ± 0.003</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>25-OH cholesterol, 48 h</td>
<td>0.049 ± 0.006</td>
<td>0.049 ± 0.004</td>
<td>0.045 ± 0.002</td>
<td>0.041 ± 0.005</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>Forskolin</td>
<td>0</td>
<td>2 ± 3</td>
<td>2 ± 3</td>
<td>2 ± 3</td>
<td>15 ± 5</td>
</tr>
<tr>
<td>+TGFβ1</td>
<td>10</td>
<td>10 ± 4</td>
<td>10 ± 4</td>
<td>10 ± 4</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>4</td>
<td>8 ± 4</td>
<td>18 ± 2</td>
<td>14 ± 1 ± 8</td>
<td>20 ± 7</td>
</tr>
<tr>
<td>24 h</td>
<td>12 ± 0 ± 2</td>
<td>11.5 ± 0.6</td>
<td>16.8 ± 2</td>
<td>13.5 ± 2 ± 0</td>
<td>19 ± 6</td>
</tr>
<tr>
<td>48 h</td>
<td>11 ± 6 ± 2</td>
<td>11.0 ± 1.5</td>
<td>13.6 ± 2 ± 0</td>
<td>16 ± 6 ± 0</td>
<td>29 ± 4</td>
</tr>
<tr>
<td>17α-Hydroxylase</td>
<td>0</td>
<td>0 ± 2</td>
<td>2 ± 3</td>
<td>3 ± 1 ± 0</td>
<td>2 ± 3</td>
</tr>
<tr>
<td>24 h</td>
<td>0 ± 5 ± 0.5</td>
<td>0.5 ± 0.10</td>
<td>6 ± 0.5 ± 0.5</td>
<td>4 ± 0.4 ± 0.5</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>48 h</td>
<td>0 ± 5 ± 0.10</td>
<td>0.5 ± 0.04</td>
<td>4 ± 0.5 ± 0.5</td>
<td>6 ± 0.4 ± 0.5</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>21-Hydroxylase</td>
<td>10</td>
<td>10 ± 4</td>
<td>16 ± 4</td>
<td>14 ± 1 ± 0</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>24 h</td>
<td>5 ± 0 ± 0.8</td>
<td>5 ± 0.05</td>
<td>9 ± 1 ± 0</td>
<td>8 ± 1 ± 0</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>48 h</td>
<td>6 ± 3 ± 0.4</td>
<td>6 ± 0.5</td>
<td>8 ± 0.9 ± 0.5</td>
<td>7 ± 0.5 ± 0.5</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>11β-Hydroxylase</td>
<td>10</td>
<td>10 ± 4</td>
<td>15 ± 7 ± 0.2</td>
<td>3 ± 6 ± 0.1</td>
<td>77 ± 9</td>
</tr>
<tr>
<td>24 h</td>
<td>1 ± 9 ± 0.2</td>
<td>1.7 ± 0.4</td>
<td>7 ± 1 ± 1 ± 0</td>
<td>3 ± 7 ± 0.5</td>
<td>48 ± 8</td>
</tr>
<tr>
<td>48 h</td>
<td>2 ± 0 ± 0.2</td>
<td>2 ± 0.1</td>
<td>15 ± 7 ± 0.2</td>
<td>3 ± 6 ± 0.1</td>
<td>77 ± 9</td>
</tr>
</tbody>
</table>

NCH295R cells were treated for 24 or 48 h without (control) or with 10 μM forskolin in the presence or absence of 2 ng/ml TGFβ1. At the end of this treatment, the cells were incubated for 2 h in fresh medium containing the steroid substrates and individual enzymatic activities were measured as described in Materials and Methods. After recovery of medium, cells were solubilized in lysis buffer and analysed for protein content. Mean % inhibition ± S.E.M. of enzymatic activity in TGFβ1-treated cells compared with untreated cells as determined from the results of three independent experiments is shown in bold characters.
Angiotensin II and forskolin-induced aldosterone production by NCI-H295R cells is inhibited by TGFβ1

Steroid 11β-hydroxylase (P450c11) and aldosterone synthase (P450 aldo), which catalyze the last steps of the biosynthesis pathways of cortisol and aldosterone are the products of two distinct but highly homologous genes: CYP11B1 and CYP11B2 respectively. Despite a high degree of identity (93% at the nucleotide and amino acid sequence level), these two enzymes differ in their catalytic properties, their hormonal regulation and their sites of expression in the adrenal cortex. The aforementioned effect of TGFβ1 on 11β-hydroxylase prompted us to examine the regulation of aldosterone synthase activity by this cytokine. As shown in Fig. 4, aldosterone production was strongly stimulated by angiotensin II (50-fold) and to a lesser extent by forskolin (4-fold). A 24-h treatment with 2 ng/ml TGFβ1 resulted in an inhibition of angiotensin II-induced and forskolin-induced aldosterone production by 80% and 65% respectively. In the same experiment, 11β-hydroxylase activity was induced 3-fold by angiotensin II and 10-fold by forskolin whereas TGFβ1 inhibited the angiotensin II effect by 30% and the forskolin effect by 70% (Table 3). TGFβ1 inhibition of angiotensin II-induced aldosterone synthase activity was time-dependent, being detectable at 12 h of treatment and reaching a maximal 80–85% between 36 and 48 h (Fig. 5).

**Figure 3** Time-course of forskolin and TGFβ1 effects on 11β-hydroxylase activity. NCI-H295R cells were cultured for the indicated periods of time in the absence (control) or the presence of forskolin (10 μM), TGFβ1 (2 ng/ml) or both forskolin and TGFβ1. At the end of treatment, the medium was renewed and the cells were assayed for 11β-hydroxylase activity by measuring the conversion of exogenous [3H]deoxycortisol (100 μM) into [3H]cortisol over a period of 2 h as described in Materials and Methods. 11β-Hydroxylase activity was normalized to the cellular protein content. Results are the means ± S.E.M. of triplicate values from three independent experiments. The statistical significance of TGFβ compared with control values and of forskolin+TGFβ compared with forskolin values was determined using the Student's t-test: *P<0.05; **P<0.01.

Angiotensin II and forskolin-induced aldosterone production by NCI-H295R cells is inhibited by TGFβ1

We then checked the effect of TGFβ1 on CYP11B1 and CYP11B2 mRNA levels. Due to the high level of identity between these two genes, we used specific cDNA probes in an RNase protection assay to simultaneously quantitate the levels of expression of these two genes in NCI-H295R cells (Holland et al. 1993, Denner et al. 1996). The expression of actin, taken as a control housekeeping gene, was also determined in the same experiment. The results shown in Fig. 6 indicate that, under basal conditions, the level of CYP11B2 was undetectable and that of CYP11B1 was barely detectable. RNA from NCI-H295R cells treated for 9 h with angiotensin II, forskolin and/or TGFβ1 (alone or in combination) were then analyzed. No direct effect of TGFβ1 was visible on the basal CYP11B1 level. In agreement with previously published results (Holland et al. 1993), a 9-h treatment with angiotensin II strongly induced CYP11B2 mRNA expression and also elevated CYP11B1 mRNA, although more weakly. Simultaneous treatment with TGFβ1 resulted in a 70% inhibition of angiotensin II-induced CYP11B2 and
CYP11B1 mRNA levels. Forskolin induced CYP11B1 and CYP11B2 mRNA expression but, in contrast to angiotensin II, it preferentially stimulated the level of CYP11B1. Simultaneous addition of TGFβ1 resulted in an inhibition of forskolin-induced CYP11B1 (58%) and CYP11B2 (65%) mRNA levels.

**TGFβ1 decreases forskolin-induced CYP11B1 promoter activity**

To identify the cis-elements involved in TGFβ1 regulation of CYP11B1 transcription, a series of deletion constructs containing progressively shorter fragments of hCYP11B1 5'-flanking DNA were transiently transfected into NCI-H295R cells. Figure 7 illustrates the reporter activity of these constructs under basal conditions or after treatment with forskolin, TGFβ1 or both. Basal luciferase activities of the constructs containing 5'-flanking sequences from –1993 to –267 bp were similar. Further deletion to –65 bp resulted in a decreased activity, confirming that DNA between –267 and –65 is essential for basal gene expression (Wang et al. 2000). TGFβ1 treatment of NCI-H295R cells transfected with these constructs did not significantly change the basal reporter gene activity. Forskolin treatment resulted in a 2·5- to 3·5-fold increase in luciferase activity for all constructs except the shortest one.

Table 3 | TGFβ1 inhibition of forskolin- and angiotensin II (AII)-induced 11β-hydroxylase and aldosterone synthase activities. The results are expressed as means ± S.E.M. of triplicate sample determinations and this experiment is representative of three independent experiments

<table>
<thead>
<tr>
<th>11β-Hydroxylase (nmol/h/mg protein)</th>
<th>Aldosterone synthase (pmol/h/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>− TGFβ1: 1·5 ± 0·3 + TGFβ1: 1·4 ± 0·2</td>
</tr>
<tr>
<td></td>
<td>Inhibition (%): 6</td>
</tr>
<tr>
<td>All</td>
<td>− TGFβ1: 5 ± 0·4 + TGFβ1: 3·5 ± 0·4</td>
</tr>
<tr>
<td></td>
<td>Inhibition (%): 30</td>
</tr>
<tr>
<td>Forskolin</td>
<td>− TGFβ1: 3± 1·5 + TGFβ1: 4·5 ± 0·7</td>
</tr>
<tr>
<td></td>
<td>Inhibition (%): 72</td>
</tr>
</tbody>
</table>

NCl-H295R cells were stimulated for 48 h, in the presence or absence of 2 ng/ml TGFβ1, in medium containing no additive (Control), 10 nM AII or 10 μM forskolin. The amount of secreted aldosterone was normalized to the amount of cellular proteins. Results are the means ± S.E.M. of triplicate values from three independent experiments.
Simultaneous treatment of NCI-H295R cells with forskolin and TGFβ1 resulted in a 25–40% inhibition of the forskolin response except for the shortest –65 bp construct. These data suggest that DNA between –267 and –65 contains regulatory sequences that are necessary for basal expression as well as for the forskolin and TGFβ responses. This sequence contains two potential SF1-binding sites (named Ad4 and Ad5) and one near-consensus cAMP response element (Wang et al. 2000).
TGFB1 targets in human NCI-H295R cells  ·  P Liakos and others

Discussion

The regulation of adrenocortical steroidogenesis is complex. Besides pituitary ACTH and angiotensin II that function as systemic endocrine regulators, several growth factors and cytokines act as local autocrine or paracrine regulators to finely tune the hormonal message (Feige & Baird 1991). Among these autocrine regulators, TGFB1 has emerged as a potent inhibitor of corticosteroid production in adrenocortical glomerulosa and fasciculata cells from a variety of species: bovine and ovine fasciculata cells (Gupta et al. 1992) and human fetal adrenal cells (Stankovic et al. 1994). Although the existence of an autocrine TGFB1 regulatory loop has only been documented and demonstrated in the bovine species (Feige et al. 1991a, Le Roy et al. 1996), it is likely to exist in humans as well, since the expressions of both TGFB1 and its signaling receptors are quite ubiquitous. However, it is puzzling to observe that distinct steps in the corticosteroid biosynthesis pathway appear to be targeted by TGFB1 in distinct species. Since the human adrenocortical tumor cell line NCI-H295R has now become a useful model to study human adrenocortical steroidogenesis, we aimed at characterizing the effects of TGFB1 on the multiple steroidogenic pathways simultaneously expressed in these tumoral cells.

The first limiting step in the synthesis of corticosteroids is the transfer of cholesterol from the cytoplasm to the inner mitochondrial membrane where it is converted into pregnenolone by cytochrome P450 scc. Steroidogenic acute regulatory (StAR) protein is also essential to this process. We previously reported that TGFB1 partially decreases both basal and forskolin-stimulated StAR expression in NCI-H295R cells through a transcriptional mechanism requiring Smad3 activation (Brand et al. 1998b). A deletion analysis of the human StAR promoter allowed us to locate the TGFB1-response element between −150 and −85 nt upstream of the transcription start site, excluding the implication of the SF1-binding sites in this regulation (Brand et al. 2000). In the present study, we observed that supplementation of forskolin-treated cells with 25-hydroxycholesterol, a membrane-permeant analog of cholesterol, did not reduce TGFB1 inhibition by more than 10%, suggesting that another limiting step downstream of pregnenolone synthesis was deeply affected by TGFB1.

Indeed, we observed that, although several enzymatic activities along the cortisol and aldosterone biosynthetic pathways were reduced after TGFB1 treatment, the most profoundly inhibited enzymes were steroid 11β-hydroxylase and aldosterone synthase. In humans and rodents, these two enzymes are encoded by two highly similar but distinct genes (CYP11B1 and CYP11B2 respectively) whereas, in the bovine, ovine and porcine species, a single gene (CYP11B) encodes an enzyme performing 11β-hydroxylation, 18-hydroxylation and 18-oxidation (Rainey 1999). In humans and rodents, specific expression of CYP11B2 in the glomerulosa zone and CYP11B1 in the fasciculata zone is responsible, in part, for the functional zonation of the adrenal cortex. The necessity to restrain aldosterone production to the thinner layer of glomerulosa cells and to have the much larger fasciculata zone synthesizing cortisol is apparent when one considers that the amount of aldosterone needed to control salt balance is 100- to 1000-fold less than the amount of cortisol required to control sugar metabolism. The promoters of the CYP11B1 and CYP11B2 genes drive not cells (Gupta et al. 1992) and human fetal adrenal cells (Stankovic et al. 1994). Although the existence of an autocrine TGFB1 regulatory loop has only been documented and demonstrated in the bovine species (Feige et al. 1991a, Le Roy et al. 1996), it is likely to exist in humans as well, since the expressions of both TGFB1 and its signaling receptors are quite ubiquitous. However, it is puzzling to observe that distinct steps in the corticosteroid biosynthesis pathway appear to be targeted by TGFB1 in distinct species. Since the human adrenocortical tumor cell line NCI-H295R has now become a useful model to study human adrenocortical steroidogenesis, we aimed at characterizing the effects of TGFB1 on the multiple steroidogenic pathways simultaneously expressed in these tumoral cells.

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only zone-specific expression but also differential hor-
monal regulation, ACTH being the major inducer of
CYP11B1 and angiotensin II the major inducer of
CYP11B2 expressions. Mutations in CYP11B1 are
responsible for steroid 11β-hydroxylase deficiency, a
form of congenital adrenal hyperplasia characterized by hyper-
tension and androgen excess, whereas mutations in
CYP11B2 cause aldosterone synthase deficiency (Peter
et al. 1999, White 2001). Recombinations between these
two genes cause an autosomal dominant form of hyper-
tension characterized by hypertension and androgen excess.

CYP11B2 appeared to down-regulate both basal and
hormone-induced expression of CYP11B1 and CYP11B2
mRNAs. Whether this regulation occurs through tran-
scriptional or post-transcriptional mechanisms remains to
be established through mRNA stability or nuclear run-on
analyses.

Surprisingly, in human adrenal cells, both basal and
ACTH-stimulated cortisol productions were reported not
to be inhibited by TGFβ1, although 17α-hydroxylase
activity was decreased (Lebrethon et al. 1994). In NCI-
H295R cells, the inhibition of cortisol production appears
to result from a strong inhibition of 11β-hydroxylase, an
enzyme that is not affected by TGFβ1 in adult or fetal
human cells. Whether these differences result from the
tumor status of NCI-H295R cells or from experimental
discrepancies remains to be determined.

The major pathway for TGFβ1 signaling involves
receptor-dependent phosphorylation of Smad2 and/or
Smad3, association of the phospho-Smads with Smad 4
and translocation of this complex to the nucleus where it
mediates the transcriptional response through binding to
TGFβ1-response elements present in the promoters of
target genes (Massagué 1998). In many cases, transcrip-
tional coactivators associate with the Smad complex to
trigger the transcriptional response. The Smad-binding
elements of the JunB (Jonk et al. 1998) and plasminogen
activator inhibitor-1 (Dennler et al. 1998) promoters have
been identified and have been shown to contain a con-
sensus CAGAC box. Nucleotide sequence analysis of the
human CYP11B1 and CYP11B2 promoters revealed the
presence of CAGAC boxes at positions −1895 in the
CYP11B1 promoter and at positions −234 and −206 in
the CYP11B2 promoter. However, when we analyzed the
CYP11B1 promoter using deletion constructs, we observed that the TGFβ1 response, as well as the
forskolin response, required the DNA sequence −65/
−267, excluding a role for the CAGAC box at
position −1895. The possibility exists that this TGFβ
effect is mediated through a signaling pathway other than
the Smad pathway. For example, Jun kinase and p38 have
also been reported to be activated by TGFβ1 in other cell
types (Derynck et al. 2001).

So far, no adrenal pathology has been clearly associated
with dysregulations of TGFβ1 expression. However, our
results suggest that overproduction of active TGFβ1 in
adrenocortical tumors might result in the absence of steroid
secretion. Non-hypersecreting adenomas represent 85% of
human adrenal incidentalomas according to a recent
Italian survey of 1004 cases (Mantero et al. 2000). Trans-
forming growth factor β was initially purified from tumor
extracts (Todaro et al. 1981) and is present in its active
form in a number of human tumors. TGFβ1 is synthesized
also by a large number of normal tissues and secreted under
a latent form that requires extracellular activation through
still poorly characterized mechanisms (enzymatic acti-
vation by plasmin, conformational activation by thrombo-
spondins . . .) to generate the active receptor-binding
peptide (Munger et al. 1997). A recent study reporting the
distribution of TGFβ1 immunoreactivity in a series of 8
secreting and 15 non-secreting adrenal tumors concluded
that TGFβ1 expression is associated with active steroid
secretion in normal adrenal tissue and adrenocortical
adenomas (Boccuzzi et al. 1999). We do not think that
these observations contradict our hypothesis because the
distinction between latent and active forms of TGFβ1 is
quite impossible to achieve by immunohistochemical
methods and the levels of TGFβ measured in this study
probably reflect the activable fraction of TGFβ1, i.e. the
sum of latent and active TGFβ1. In our opinion, a more
extensive study using a functional method for detection of
active TGFβ1 (such as a radioreceptor assay or a TGFβ1
reporter gene assay) still has to be performed in human
adrenocortical tumors. This should allow us to challenge
our hypothesis of an association between high levels of
active TGFβ1 and reduced steroidogenic capacities.

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