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

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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 deoxycortisol 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 adrenocorticotropic (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 tumors, NCI-H295R is a useful experimental model since it is the only steroid-secreting human adrenocortical tumor cell line presently available. It turns out to be a unique model to simultaneously study the effects of any cytokine on the three steroid synthesis pathways that are normally separated in glomerulosa, fasciculata and reticularis cells. The observations that NCI-H295R cells overexpress insulin-like growth factor II (IGF-II), a genetic abnormality currently observed in human adrenocortical carcinoma, and are IGF-II-dependent for growth also support their use as a good in vitro model of adrenocortical 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 [1,2,3-3H]11-deoxycortisol (41.7 Ci/mmol) were from NEN Life Science Products (Zaventem, Belgium). [1,2,6,7-3H]Corticosterone (60 Ci/mmol), [1,2,6,7-3H]cortisol (100 Ci/mmol) and [1,2,6,7-3H]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-Salpetrière, 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 (section 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 quantitatively referred 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 RIAs. Steroid 17α-hydroxylase activity was assayed by measuring the transformation of [3H]pregnenolone into [3H]17α-hydroxyprogrenolone. 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 was analyzed by thin layer chromatography on silica gel plates (F-254; Merck) in chloroform:ethyl acetate (1:1, v/v). Individual enzymatic activities

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3β-Hydroxysteroid dehydrogenase/isomerase activity was measured by incubating the cells with [1,2,6,7-3H]dehydroepiandrosterone (DHEA) (100 μM) in the presence of 2 μM metyrapone (an inhibitor of 11β-hydroxylase). Radiolabeled dehydroepiandrosterone and androstenedione were separated by thin-layer chromatography in chloroform:acetone (9:1, v/v) and their radio-active content was measured as described above.

Steroid 21-hydroxylase activity was measured in the presence of metyrapone (2 μM) using [3H]17-hydroxyprogesterone (50 μM) as a substrate. [3H]17-Hydroxyprogesterone and [3H]deoxycorticosterone were separated by TLC 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]deoxycorticosterone 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 MAXscript 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 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 TGFB1 (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-hydroxyandrostenedione 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-hydroxyandrostenedione 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 17α-hydroxyprogesterone (20 \( \mu \)M). 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-hydroxylase 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\textsubscript{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-hydroxyprogrenenolone (17-OH pregnenolone) or 17α-hydroxyprogesterone (17-OH 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-hydroxyprogrenenolone 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.

**Figure 2** Time-course of forskolin and TGFβ1 effects on cortisol production. 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 replaced by fresh medium and cortisol secreted over a further 2-h incubation period was measured by RIA. The amount of secreted cortisol was normalized to the amount of cellular proteins. Results are the means ± S.E.M. of triplicate values from three independent experiments. The statistical significance of TGFβ compared with control values and that of forskolin + TGFβ compared with forskolin values was determined using the Student’s t-test: *P<0.05; **P<0.01.

**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.

aPercentage 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>
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<tr>
<td>Cholesterol side chain cleavage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol, 48 h</td>
<td>0.036 ± 0.007</td>
<td>0.034 ± 0.004</td>
<td>5</td>
<td>7 ± 2</td>
<td></td>
</tr>
<tr>
<td>25-OH cholesterol, 48 h</td>
<td>0.049 ± 0.006</td>
<td>0.049 ± 0.004</td>
<td>0</td>
<td>4 ± 4</td>
<td></td>
</tr>
<tr>
<td>3β-HSD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>12.0 ± 2</td>
<td>11.5 ± 0.6</td>
<td>4</td>
<td>8 ± 4</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>11.6 ± 2</td>
<td>10.0 ± 1.5</td>
<td>10</td>
<td>10 ± 4</td>
<td></td>
</tr>
<tr>
<td>17α-Hydroxylase</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>24 h</td>
<td>0.5 ± 0.05</td>
<td>0.5 ± 0.10</td>
<td>0</td>
<td>0 ± 2</td>
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<tr>
<td>48 h</td>
<td>0.5 ± 0.10</td>
<td>0.5 ± 0.04</td>
<td>0</td>
<td>2 ± 3</td>
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<tr>
<td>21-Hydroxylase</td>
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<tr>
<td>24 h</td>
<td>5.0 ± 0.8</td>
<td>5.3 ± 0.5</td>
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<tr>
<td>48 h</td>
<td>6.3 ± 0.4</td>
<td>6.0 ± 0.7</td>
<td>4</td>
<td>2 ± 3</td>
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<tr>
<td>11β-Hydroxylase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>1.9 ± 0.2</td>
<td>1.7 ± 0.4</td>
<td>10</td>
<td>6 ± 5</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>2.0 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>0</td>
<td>4 ± 5</td>
<td></td>
</tr>
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</table>
| NCI-H295R 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 and/or TGFβ2. 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 and aldosterone synthase activities were measured. The 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).

TGFβ1 down-regulates CYP11B1 and CYP11B2 mRNA expression

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></th>
<th>11β-Hydroxylase</th>
<th>Aldosterone synthase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol/h/mg protein)</td>
<td>(pmol/h/mg protein)</td>
</tr>
<tr>
<td>Control</td>
<td>1·5 ± 0·3</td>
<td>0·75 ± 0·06</td>
</tr>
<tr>
<td>All</td>
<td>1·4 ± 0·2</td>
<td>0·65 ± 0·09</td>
</tr>
<tr>
<td>Forskolin</td>
<td>3·5 ± 0·4</td>
<td>3·0 ± 0·4</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>3·5 ± 0·4</td>
<td>5·7 ± 1·2</td>
</tr>
<tr>
<td>TGFβ1 + AII</td>
<td>4·5 ± 0·7</td>
<td>9·9 ± 0·9</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. At the end of this treatment, the cells were incubated for 2 h in fresh medium containing [3H]deoxycortisol (11β-hydroxylase activity) or corticosterone (aldosterone synthase activity). 11β-Hydroxylase and aldosterone synthase activities were then determined as described in Materials and Methods and normalized to cellular protein content. Percentage inhibition of cortisol production observed in TGFβ1-treated cells compared with untreated cells in this experiment is shown.

Figure 4 Effect of forskolin, angiotensin II and TGFβ1 on aldosterone secretion. NCI-H295R cells were cultured for 24 h in the absence (control) or the presence of 10 μM forskolin, 10 nM angiotensin II (AII), 2 ng/ml TGFβ1, both angiotensin II and TGFβ1 or both forskolin and TGFβ1. At the end of treatment, the medium was replaced by fresh medium and aldosterone secreted over a further 4-h incubation period was measured by RIA. 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.

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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).

Figure 5  Time-course of angiotensin II and TGFβ1 effects on aldosterone synthase activity. NCI-H295R cells were cultured for the indicated periods in the absence (control) or presence of angiotensin II (AII; 10 nM), TGFβ1 (2 ng/ml) or All and TGFβ1. At the end of the indicated time, media were removed and cells were assayed for aldosterone synthase activity by the metabolism of exogenous corticosterone (10 μM). Medium was recovered and aldosterone secreted in the medium was quantitated by RIA. Aldosterone synthase activity was normalized to the amount of cellular proteins. 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.

Figure 6  RNase protection analysis of CYP11B1 and CYP11B2 mRNA levels under forskolin, angiotensin II or TGFβ1 treatment. NCI-H295R cells were treated for 9 h without (Ctl) or with 10 nM angiotensin II (AII), 10 μM forskolin (Forsk), 2 ng/ml TGFβ1, or the indicated combinations. Total RNA was extracted from these cells, hybridized with 32P-labeled CYP11B1 and CYP11B2 probes and digested by ribonuclease. Simultaneous hybridization with a 32P-labeled actin probe was used for RNA loading control. Protected RNA species were examined by electrophoresis on denaturing 5% acrylamide gels. This experiment is representative of two independent experiments. M, DNA markers.
means value obtained with the over renilla luciferase activities and are normalized to the basal were measured. The results are expressed as the ratios of firefly and TGF effectors: forskolin (10
transfected cells were treated for an additional 24 h with various
transfected with a renilla luciferase plasmid. After 24 h of culture,
electroporation with luciferase (Luc) reporter gene constructs
containing progressive deletions of human
The regulation of adrenocortical steroidogenesis is com-
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function as systemic endocrine regulators, several growth
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inhibitory (StAR) protein is also essential to this
process. We previously reported that TGFβ1 partially
decreases both basal and forskolin–stimulated StAR
expression in NCI-H295R cells through a transcrip-
tional mechanism requiring Smad3 activation (Brand et al. 1998b). A deletion analysis of the human StAR promoter
allowed us to locate the TGFβ1–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 TGFβ1
inhibition by more than 10%, suggesting that another
limiting step downstream of pregnenolone synthesis was
deeply affected by TGFβ1.
Indeed, we observed that, although several enzymatic
activities along the cortisol and aldosterone biosynthetic
pathways were reduced after TGFβ1 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 (CYP11B1) 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 neces-
sity to restrain aldosterone production to the thinner layer
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biosynthesis pathway appear to be targeted by TGFβ1 in
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Discussion
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(Feige et al. 1986, Rainey et al. 1988), bovine glomerulosa

Figure 7 Regulation of human CYP11B1 promoter by forskolin and TGFβ1. NCI-H295R cells were transiently transfected by
electroporation with luciferase (Luc) reporter gene constructs
containing progressive deletions of human CYP11B1 5′-flanking
DNA. Together with this firefly reporter construct, cells were
transfected with a renilla luciferase plasmid. After 24 h of culture,
transfected cells were treated for an additional 24 h with various
effectors: forskolin (10 μM), TGFβ1 (2 ng/ml) and both forskolin
and TGFβ1. The cells were then lysed and the luciferase activities
were measured. The results are expressed as the ratios of firefly
over renilla luciferase activities and are normalized to the basal
value obtained with the –1993 construct. They represent the
means ± S.E.M. of data from three independent experiments.

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only zone-specific expression but also differential hormonal 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 hypertension 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 hyperaldosteronism (Pascoe et al. 1992). Recently, congenital adrenal hyperplasia and steroid 11β-hydroxylase deficiency were observed in a patient with homozygous deletions of the CYP11B1 and CYP11B2 genes (Portrat et al. 2001).

Hormonal regulation of CYP11B1 and CYP11B2 gene expression by forskolin and angiotensin II in NCI-H295R cells was consistent with previous reports, with forskolin preferentially inducing CYP11B1 and angiotensin II preferentially inducing CYP11B2 (Denner et al. 1996). TGFβ1 appeared to down-regulate both basal and hormone-induced expression of CYP11B1 and CYP11B2 mRNAs. Whether this regulation occurs through transcriptional 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 tumoral 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, transcriptional 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 consensus 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 TGFβ1 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 (Derynick et al. 2001).

So far, no renal 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). Transforming 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 activation by plasmin, conformational activation by thrombospondins. . . ) 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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