Expression of activin/inhibin signaling components in the human adrenal gland and the effects of activins and inhibins on adrenocortical steroidogenesis and apoptosis

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Abstract

Activins and inhibins are structurally related glycoprotein hormones modulating pituitary FSH secretion and gonadal steroidogenesis. Activins and inhibins are also produced in the adrenal cortex where their physiological role is poorly known. Hormonally active human adrenocortical tumors express and secrete inhibins, while in mice adrenal inhibins may function as tumor suppressors. To clarify the significance of adrenal activins and inhibins we investigated the localization of activin/inhibin signaling components in the adrenal gland, and the effects of activins and inhibins on adrenocortical steroidogenesis and apoptosis.

Activin receptor type II/IIB and IB, activin signal transduction proteins Smad2/3, and inhibin receptor beta-glycan were expressed throughout the adrenal cortex, whereas Smad4 expression was seen mainly in the zona reticularis and the innermost zona fasciculata as evaluated by immunohistochemistry. Treatment of cultured adrenocortical carcinoma NCI-H295R cells with activin A inhibited steroidogenic acute regulatory protein and 17α-hydroxylase/17,20-lyase mRNA accumulation as evaluated by the Northern blot technique, and decreased cortisol, androstenedione, dehydroepiandrosterone and dehydroepiandrosterone sulfate secretion as determined by specific enzyme immunoassays. Activin A increased apoptosis as measured by a terminal deoxynucleotidyl transferase in situ apoptosis detection method. Inhibins had no effect on steroidogenesis or apoptosis.

In summary, activin/inhibin signaling components are coexpressed in the zona reticularis and the innermost zona fasciculata indicating full signaling potential for adrenal activins and inhibins in these layers. Activin inhibits steroidogenic enzyme gene expression and steroid secretion, and increases apoptosis in human adrenocortical cells. Thus, the activin–inhibin system may have a significant role in the regulation of glucocorticoid and androgen production and apoptotic cell death in the human adrenal cortex.


Introduction

Activins and inhibins are members of the transforming growth factor β (TGFβ) superfamily of growth and differentiation factors. They are structurally related glycoproteins composed of two of the three different subunits (βA;βA, βA;βB, βB;βB; activin A, activin AB and activin B; α;βA, α;βB; inhibin A and inhibin B respectively). Gonads are the most important sources of inhibins in humans (reviewed in Vale et al. 1988, Ying 1988). Gonadal inhibins suppress pituitary follicle-stimulating hormone secretion and stimulate ovarian theca cell androgen production (reviewed in Findlay 1993). Activins are widely expressed and they have been shown to regulate a variety of cell functions, including steroid hormone production and apoptosis (reviewed in Woodruff 1998). Activins and inhibins are also produced in adrenal cells (Voutilainen et al. 1991, Spencer et al. 1992, Voutilainen 1995, Vänttinen et al. 2002).

In the adrenals, inhibins were originally suggested to be tumor-suppressor proteins on the basis that inhibin α-subunit knockout mice developed malignant adrenal tumors (Matzuk et al. 1994). However, later studies showed that inhibins do not have marked tumor-suppressor activity in human adrenals, although loss of immunoreactivity was reported in a subgroup of adrenocortical carcinomas (Munro et al. 1999). In contrast, increased inhibin α-subunit mRNA and peptide expression, and increased secretion of immunoreactive inhibins, were reported in virilizing and, to some extent, in...

Adrenocorticotropin (ACTH) regulates adrenal inhibin/activin production suggesting that inhibins and activins may mediate or modulate the effects of ACTH in a para/autocrine manner (Voutilainen et al. 1991, Vänttinen et al. 2002). ACTH regulates steroidogenesis at multiple levels. In addition to its acute effect on steroid secretion, it increases the production of the steroidogenic acute regulatory protein (StAR) enabling the transport of cholesterol into mitochondria to be metabolized to steroid hormones (reviewed in Stocco 2001). ACTH stimulates the synthesis of steroidogenic enzymes, including 17α-hydroxylase/17,20-lyase (P450c17) essential for glucocorticoid and androgen production (reviewed in Miller 1988). The role of adrenal activins in the regulation of steroidogenesis is controversial; they have been shown to stimulate ACTH-induced cortisol secretion in human fetal adrenal cells (Spencer et al. 1992), but to inhibit ACTH-induced cortisol and dehydroepiandrosterone (DHEA) secretion in bovine adrenal cells (Nishi et al. 1992). Inhibins had no effects on adrenal steroidogenesis in these studies.

The signaling system of activins and inhibins in the adrenal gland is poorly characterized. Activins mediate their effects through specific receptors on cell membranes and via a cascade of cytosolic Smad proteins (reviewed in Itoh et al. 2000, Pangas & Woodruфф 2000). In brief, both type I and II receptors (ActRI/IB and ActRII/IIB respectively), receptor-regulated Smads (R–Smads; Smad2 and Smad3) and a common-mediator Smad (Co-Smad; Smad4) are needed for successful activin signaling. In addition, inhibitory Smads (I–Smads; Smad6 and Smad7) regulate activin signaling. Inhibins, in turn, antagonize activin signaling through binding to ActRII co-receptor betaglycan (βG, also known as TGFβ type III receptor; reviewed in Gray et al. 2002). It is known that radiolabeled activin and inhibin bind to rat adrenals and that activin and inhibin receptor mRNAs are expressed in human adrenal cells (Woodruфф et al. 1993, Vänttinen et al. 2002).

Adrenocortical function is determined by both the number and the steroidogenic activity of the adrenocortical cells. The rate of cell proliferation and apoptosis contribute to the cell number. In this study we wanted to shed more light on the role of activins and inhibins in the regulation of both adrenal steroidogenesis and apoptosis. Activin and inhibin receptor and Smad signal transduction peptide localizations in the adrenal cortex were studied by immunohistochemistry. Human adrenocortical carcinoma cell line NCI-H295R, expressing all adrenal steroidogenic enzymes, secreting glucocorticoids and androgens (Gazdar et al. 1990, Staels et al. 1993, Rainey et al. 1994), and expressing activin/inhibin receptor genes (Vänttinen et al. 2002) was used as a model for studying the effects of activins and inhibins in adrenocortical cell steroidogenesis and apoptosis. 8-Bromo cAMP (8-BrcAMP), a protein kinase A activator, was used to mimic the effects of ACTH on adrenocortical steroidogenesis as the NCI-H295R cell line does not express functional ACTH receptors.

Materials and Methods

Ethical considerations

The Research Ethics Committees of the Kuopio and Helsinki University Hospitals approved the study protocols, and the patients gave informed written consent.

Immunohistochemistry

Normal adult adrenal tissues were obtained during surgery for renal tumors. Immunostaining was performed on 5 µm sections cut from paraffin-embedded blocks of paraformalin-fixed tissues. The sections were deparaffinized in xylene, rehydrated in a series of graded alcohols, and permeabilized by microwaving at high power for 5 min three times in citrate buffer (10 mM Na citrate, pH 6·0). The sections were then washed in PBS and nonspecific staining was blocked with normal rabbit serum for at least 30 min at room temperature. Endogenous peroxidase activities were blocked by 1% H2O2 for 30 min. The antigens in the sections were detected with corresponding affinity-purified goat polyclonal antibodies (ActRII/IIB, sc-5669; ActRIIB, sc-11986, Smad2/3, sc-6032; Smad4, sc-1908, TGFβRIII (βG), sc-6199; all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; and another TGFβRIII antibody from Research Diagnostics, Inc., Flanders, NJ, USA), which were applied at 1:100 dilution. The primary antibodies were added on the adrenal sections in PBS, and incubation was performed overnight at 4 °C. After this incubation, the slides were washed in PBS three times. The primary antibodies were identified using biotin-conjugated rabbit anti-goat IgG from an ABC-Elite Kit (PK-6105; Vector Laboratories, Inc., Burlingame, CA, USA), followed by incubation with ABC solution according to the manufacture’s instructions. Finally, light counterstaining was performed with hematoxylin, and the sections were dehydrated and mounted. Each staining was repeated with at least five different sections with similar results. For the negative controls, the primary antibodies were replaced by normal goat serum or PBS alone. To exclude the effect of possible endogenous biotin, biotin blocking (avidin-biotin blocking kit; Vector Laboratories) was performed in two samples for each antigen detection before the addition of the primary antibodies.

Cell cultures

NCI-H295R human adrenocortical cell line was obtained from American Type Culture Collection (Manassas, VA, USA). For the steroid and mRNA studies the cells were
plated at a density of $1 \times 10^6$ cells per well on 35 mm plastic culture dishes (Nunc, Roskilde, Denmark). The medium was DMEM-F12 containing 2% Ultroser (Life Technologies, Paisley, Strathclyde, UK), ITS+1 liquid media supplement (Sigma, St Louis, MO, USA), penicillin (100 IU/ml), streptomycin sulfate (100 µg/ml) and glutamine (0.5 mM). Cell cultures were grown as monolayers at 37°C in a 95% air–5% CO2 humidified environment and the culture media were changed every 2–3 days. The viability of the cell cultures was monitored by phase contrast light microscopy.

Experimental procedures were performed when the cell cultures had reached confluency. In the experiments the cultured cells were incubated for 48 h with or without 8-BrcAMP (Sigma), recombinant human activin A, inhibin B (both from R&D Systems, Minneapolis, MN, USA), inhibin A (generously provided by Dr A F Parlow from NIDDK National Hormone and Pituitary Program, NIH, Bethesda, MD, USA), or cycloheximide (inhibitor of protein synthesis; Sigma). After the experiments the culture media were collected and stored at $-70°C$.

**RNA and DNA preparation**

Fetal adrenals were obtained from legal abortions performed for social or medical reasons. Normal adult adrenal tissues were obtained during surgery for renal tumors. RNA was extracted by ultracentrifugation through a cesium chloride cushion. The cultured cells were lysed using Trizol Reagent (Life Technologies) according to the manufacturer’s protocol. Cell lysates were separated by chloroform into an aqueous phase containing RNA and an organic phase containing proteins and DNA. Trace amounts of genomic DNA were removed from the RNA-containing phase by treatment with Rnase-free Dnase (Promega Corp., Madison, WI, USA). RNA was precipitated from the aqueous phase overnight with equal volume of isopropanol, washed once with 75% ethanol, air-dried, and dissolved in Rnase-free water. DNA was extracted from the organic phase by incubation with 10% ethanol and centrifugation. DNA-pellet was then washed twice with 0.1 M Na citrate/10% ethanol, and once with 75% ethanol, air-dried, and dissolved in 10 mM Tris/1 mM EDTA solution with pH adjusted to 8.0 with NaOH. RNA and DNA were stored at $-70°C$ if not used immediately for further analyses.

**RT-PCR**

The RT-PCR techniques were as described previously (Vänttinen et al. 2002). Briefly, 1 µg total RNA, oligo(dT)$_{20}$ primer (Amersham, Little Chalfont, Bucks, UK), four dNTPs (Amersham), avian myeloblastosis virus reverse transcriptase (Finnzymes, Espoo, Finland), and RT reaction buffer (Finnzymes) were used for cDNA synthesis. The amplification primers used for the PCR of human Smads and the expected lengths of the PCR products are shown in Table 1. PCR was performed by combining one-tenth of the RT mixture with GeneAmp PCR buffer (Roche Molecular Systems, Branchburg, NJ, USA), dNTP mixture (Amersham), 3’- and 5’-oligonucleotide primers (TAG Copenhagen, Copenhagen, Denmark) and AmpliTaq Gold DNA polymerase (Roche Molecular Systems). The samples were then cycled (X × 40) using Hybaid PCR Express thermal cycler (Ashford, Middlesex, UK). Aliquots of the PCR reaction products were size-fractionated in 3% agarose gel (Prondisa, Alcobendas, Spain). PCR products were sequenced with an automatic DNA analysis system to confirm their specificity (ABI Prism 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA).

**Northern blot analysis**

A Northern blot sample consisted of 20 µg total RNA in $H_2O$, 10 × MOPS, deionized formamide, 37% formaldehyde and ethidium bromide. Samples were size-fractioned in denaturing 1% agarose gel (Prondisa). After the electrophoresis, RNA was transferred onto Hybond-N+ nylon filters (Amersham), and fixed by UV cross-linking. The prehybridization and hybridization techniques were

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<thead>
<tr>
<th>Gene</th>
<th>5’ Sense</th>
<th>3’ Antisense</th>
<th>PCR product (bp)</th>
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<tbody>
<tr>
<td>Smad2</td>
<td>5’-TCTTACTACTCTTTCCCCCTG-3’ (805-824)</td>
<td>5’-GGTTCTCCAACCCCTGTAGT-3’ (903-921)</td>
<td>117</td>
</tr>
<tr>
<td>Smad3</td>
<td>5’-GCTGGTCAGTGTGCTTAG-3’ (1325-1344)</td>
<td>5’-AATGGGTTGAGTAGAGTC-3’ (1411-1430)</td>
<td>106</td>
</tr>
<tr>
<td>Smad4</td>
<td>5’-GGTGAGGTAATAGTGCC-3’ (599-618)</td>
<td>5’-AATGGGTTGAGTAGAGTC-3’ (1411-1430)</td>
<td>106</td>
</tr>
<tr>
<td>Smad6</td>
<td>5’-GCTACACCTCCCTCATCAC-3’ (1849-1868)</td>
<td>5’-GGTGGTACACCCCGTAGAG-3’ (1972-1990)</td>
<td>142</td>
</tr>
<tr>
<td>Smad7</td>
<td>5’-GAAGTGAGGGCTGTGTTG-3’ (812-831)</td>
<td>5’-CGGATATCGAGTAAAGG-3’ (921-940)</td>
<td>129</td>
</tr>
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used as described previously (Liu et al. 1994). The StAR mRNA was detected with an oligonucleotide probe (Liu et al. 1996), P450c17 mRNA (Chung et al. 1987) and 28S rRNA (loading control; Arneheim 1979) with cDNA probes. Hybridized filters were autoradiographed and the hybridization signals quantified by densitometric scanning.

Enzyme immunoassays for steroid hormones

Steroid hormones were analyzed by competitive enzyme immunoassays. A cortisol measurement kit was purchased from Diagnostic Products Corporation (Los Angeles, CA, USA; product code DKCO01), Androstenedione, DHEA and DHEA-S (DHEA sulfate) measurement kits were purchased from Diagnostic System Laboratories (Webster, TX, USA; product codes DSL–10–3800, DSL–10–9000 and DSL–10–3500 respectively). The detection limits of the cortisol, androstenedione, DHEA and DHEA-S assays were 8·3, 0·1, 0·3 and 40·7 nM respectively. The intra- and interassay coefficients of variation were respectively 4·4 and 6·7%, 8·8 and 7·5%, 9·9 and 5·4%, and 5·1 and 7·2%.

Apoptosis analysis

A TACS terminal deoxynucleotidyl transferase (TdT) in situ apoptosis kit (R&D Systems) was used for detecting apoptosis in cultured NCI-H295R cells. Briefly, the NCI-H295R cells were plated on chamber glass slides (Nunc) at a density of 0·2 × 10⁶ per each 0·8 cm² well and treated with activin A for 48 h. The slides were fixed in cold methanol for 5 min and permeabilized in Cytonin solution (R&D Systems) for 60 min. Then TdT labeling buffer containing TdT-dNTPs, Mn⁺⁺ and TdT enzyme was added on slides for 60 min incubation at 37 °C. The labeling was stopped after incubation by TdT stop buffer for 5 min. The slides were washed twice in deionized water (dH₂O), incubated in streptavidin-horseradish peroxidase for 10 min, washed again twice in dH₂O, and finally incubated in TACS Blue Label for 2–5 min. The slides were processed through increasing concentrations of ethanol, then xylene and finally covered with Depex (BDH Laboratory Supplies, Poole, Dorset, UK). Positive and negative cell control slides provided by R&D Systems, and a properly stained slide without TdT enzyme were used. The cells were considered to be apoptotic when the bluish color for nuclear apoptosis was seen and the morphological criteria for apoptosis were fulfilled (Gorczyca et al. 1993). The apoptotic index (%) was defined as a ratio between the apoptotic and apoptotic plus non-apoptotic cells determined from six randomly counted non-overlapping microscope fields per well with × 400 magnification with an Olympus BH-2 microscope equipped with a 0·2 × 0·2 mm ocular grid (Olympus Optical Company, Hamburg, Germany). NCI-H295R cell apoptosis was studied also by the DNA fragmentation analysis described previously (Zhu & Wang 1997).

Statistical analyses

Single experiments consisted of several hormonal or other manipulations each on one to three separate dishes. The steroid concentrations of the cell culture media were normalized with the DNA content of the respective dishes, and the scanning values of StAR and P450c17 mRNA signals with the respective 28S rRNA values. Results are shown as arithmetical means ± S.E.M. in relation to the control adjusted to 100%. The statistical significances were estimated using the Mann–Whitney test in two-group comparisons or the Kruskal–Wallis test in multiple comparisons followed by a Mann–Whitney test with Bonferroni’s correction as a post hoc test. The level of significance was chosen as P<0·05.

Results

Activin/inhibin receptors and intracellular activin signaling peptides and mRNAs are expressed in the human adrenal cortex

Human adrenal cortex showed differential staining pattern for the activin/inhibin signaling system components. ActRII/IIB was expressed throughout the adrenal cortex and medulla, but the staining was most intense in the zona reticularis (ZR) and the innermost zona fasciculata (ZF). ActRIIB and Smad2,3 were coexpressed throughout the adrenal cortex with the most intense staining in the zona glomerulosa (ZG), and very faint staining in the medulla. Smad4 showed minimal or no expression in the ZG, moderate expression in the medulla and strong expression in the ZR and the innermost ZF. JβG was expressed throughout the adrenal cortex (Fig. 1). Its expression was relatively weak but consistent with two different antibodies. Human fetal and adult adrenal, and NCI-H295R carcinoma cells expressed Smad2–4 and Smad6–7 mRNAs as determined by RT-PCR, although Smad2 expression in fetal cells was very weak (Fig. 2).

8-BrAMP stimulates whereas activin A inhibits StAR and P450c17 gene expression

StAR and P450c17 specific hybridizations of Northern blot filters revealed single mRNA bands with relatively strong signals. 8-BrAMP (0·1 mM) induced parallel StAR and P450c17 mRNA accumulation in NCI-H295R cells (at 48 h up to 120% of control, P<0·05; Fig. 3). Activin A inhibited time- and dose-dependently StAR and P450c17 mRNA accumulation. This inhibitory effect was relatively slow and significant for both genes after
48 h incubation at activin concentrations of 30–100 ng/ml. Activin A decreased StAR and P450c17 mRNA accumulation maximally down to 60–80 and 40–70% of control respectively (P<0.05 for both, n=11; Figs 3–5). Activin A inhibited totally the stimulatory effect of 8-BrcAMP on P450c17, and partially that on StAR gene expression (P<0.05; Fig. 3). Inhibin A or inhibit B had no effects on StAR or P450c17 expression (1–100 ng/ml, 48 h for both; Fig. 3; inhibit B data not shown). Cycloheximide (an inhibitor of protein synthesis) inhibited StAR but increased P450c17 mRNA accumulation (P<0.05 for both). Cycloheximide enhanced the inhibitory effect of activin A on StAR expression but inhibited it on P450c17 expression (Fig. 5).

Figure 1 Immunohistochemical localization of activin/inhibin signaling components in the adrenal cortex. Human adrenal sections were stained with antibodies detecting both activin receptor type II and IIB (ActRII/IIB), activin receptor type IB (ActRIB), both Smad2 and Smad3 (Smad2,3), and Smad4 and betaglycan (BG). Each panel shows both cortex and medulla (magnification × 100), except the BG staining on the right bottom showing only the adrenal cortical layers (magnification × 200). Positive staining for each antigen is seen in red. Nuclei are counterstained with hematoxylin, producing a light blue color. ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis; M, medulla.
8-BrcAMP stimulates and activin A inhibits steroid secretion

Steroid secretion by cultured NCI-H295R cells increased in relation to the cell number and reached maximum when the cultures became confluent. At that stage the basal secretion rates of cortisol, androstenedione, DHEA and DHEA-S were 72.2 ± 7.0, 23.4 ± 3.9, 0.8 ± 0.2 and 33.7 ± 2.2 nmol/mg DNA per 48 h respectively.

8-BrcAMP (0.1 mM, 48 h) stimulated cortisol, androstenedione, DHEA and DHEA-S secretion up to 240, 210, 150 and 110% of control (P<0.05 for all, n=4; Fig. 6). Activin A inhibited the secretion of all measured steroids dose- and time-dependently (1–100 ng/ml, 6–48 h; Fig. 7). The inhibition became statistically significant at 24–48 h. At 48 h activin A inhibited cortisol, androstenedione, DHEA and DHEA-S production in different experiments down to 60–80% of control (P<0.05, n=12; Figs 6 and 7). Inhibin A (Fig. 6) or inhibin B (0–100 ng/ml both, 48 h) had no significant effect on steroidogenesis (inhibin B data not shown).

When NCI-H295R cells were incubated for 48 h with a combination of 8-BrcAMP and activin A, the secretion of cortisol, androstenedione and DHEA-S decreased down to 76, 45, and 66% of the levels secreted by merely 8-BrcAMP-treated cells (P<0.05 for all, n=4; Fig. 6). Activin A inhibited 8-BrcAMP-induced DHEA secretion only transiently (6–36 h, not shown), but at 48 h it increased it up to 116% (P<0.05, n=4). Inhibin A (Fig. 6) or inhibin B (40 ng/ml for both) did not interfere with the effects of 8-BrcAMP (data not shown for inhibin B).

Activin A increases apoptosis

Phase contrast light microscopy revealed that activin A treatment induced apoptotic morphology in NCI-H295R cells. TdT labeling showed that activin A induced apoptosis dose-dependently and this effect was observable after 24 h of treatment. Activin A treatment (30 ng/ml, 48 h) increased the apoptotic index of NCI-H295R cells up to 290% of control (P<0.05, n=5; Fig. 8). DNA fragmentation analysis confirmed the apoptotic effect of activin A (data not shown). Inhibins had no effect on NCI-H295R apoptosis (data not shown).

Discussion

We have shown that: (i) activin/inhibin receptors and Smad signal transduction peptides are coexpressed in the innermost ZF and the ZR of the adult adrenal gland; (ii) Smad genes are expressed in fetal and adult adrenal cells; (iii) activin A treatment decreases basal and cAMP-induced StAR and P450c17 mRNA accumulation in cultured adrenocortical NCI-H295R cells; (iv) activin A inhibits the secretion of cortisol, androstenedione, DHEA and DHEA-S in NCI-H295R cells; and (v) activin A induces apoptotic cell death in NCI-H295R cells.

This and previous studies (Vänttinen et al. 2002) showed that human adrenocortical cells express mRNAs for the activin/inhibin receptors and the R-, Co-, and
I-Smads. The coexpression of these components in the adrenal cortex confirms the presence of a functional activin/inhibin signaling system in the adult adrenal gland. Despite the expression of activin receptors and R-Smads throughout the cortex, the expression of Co-Smads seems to limit activin signaling mainly to the ZR and the innermost ZF. The significance of differential expression of activin receptors and Smads in the adrenocortical zones is not known but it may represent a regulatory mechanism for activin actions. Interestingly, the innermost ZF and ZR have previously been shown to express inhibin α- and β-subunit peptides (Munro et al. 1999, Arola et al. 2000). In connection with βG expression, this suggests that adrenal inhibins may interfere with activin signaling in the adrenal gland. In addition, the I-Smads may also regulate activin signaling in the adrenal cortex. The expression of activin signaling system in the ZR and ZF suggests a role for adrenal activins in glucocorticoid and androgen production. In our study activin treatment reduced both cortisol and androgen production in NCI-H295R cells. Similarly, a previous report (Nishi et al. 1992) shows that activin A inhibits ACTH-induced cortisol and DHEA secretion in bovine adrenal cells. A similar connection between steroidogenesis and activins has been reported in primary cultures of adrenal cells where spontaneously increasing activin A gene expression is associated with decreasing steroid production and inhibin α-gene expression (Voutilainen et al. 1991). In contrast, one study suggested that activin A stimulates ACTH-induced cortisol secretion in human fetal, but not in adult adrenal cells (Spencer et al. 1992). These discrepancies could be explained by the different developmental stage of the cells used in these studies, which has been reported to influence the response to activin treatment (Miró & Hillier 1992, Di Simone et al. 1996). Relatively high activin concentration was needed to cause effects on NCI-H295R cells. This may be explained by the high endogenous follistatin (an activin-binding protein) production in these cell cultures (T Vänttinen, J Liu, T Kuulasmaa, P Rasmus and R. Voutilainen, unpublished observations).

Activins seem to regulate steroidogenesis at the transcriptional level. In human ovarian theca-like tumor cells activin decreased forskolin-induced P450c17 (Sawetawan et al. 1996) and StAR expression (Dooley et al. 2000). In our study activins decreased P450c17 and StAR gene expression.
expression also in adrenocortical cells. The effects of activins are very similar to those of TGFβ1, another member of the TGFβ superfamily and a well-known inhibitor of adrenal steroidogenesis (reviewed in Chambaz et al. 1996). This is not surprising as TGFβs share a common Smad pathway with activins. TGFβ1 has been reported to decrease both human adrenal P450c17 (Lebrethon et al. 1994) and StAR (Brand et al. 1998) mRNA expression. Thus, it seems that the human adrenal Smad pathway is anti-steroidogenic and can be activated by several para/autocrine peptides, including activins and TGFβs.

Stimulation of androgen production in ovarian theca cells in response to inhibins (reviewed in Findlay 1993) and high inhibin α-subunit mRNA and peptide expression in adrenal virilizing tumors (Nishi et al. 1995, Arola et al. 2000, Vänttinen et al. 2002), suggest that inhibins might stimulate adrenal androgen production. On the other hand, inhibins have not been found to have any direct effects in adrenal cells. Inhibins could regulate adrenal function via two indirect mechanisms. First, the expression of the inhibin receptor βG in adrenal cells suggests that inhibins could antagonize the effects of activins. Secondly, each dimeric inhibin molecule produced limits the availability of β-subunits for activin synthesis. The parallel effects of ACTH on steroidogenesis and inhibin production on the one hand, and increasing activin production and decreasing steroidogenesis in the absence of ACTH on the other hand support these hypotheses (Voutilainen et al. 1991, Vänttinen et al. 2002).

Figure 5 The effect of cycloheximide (CH; 20 µg/ml), activin A (Act; 20 ng/ml), and their combination on StAR and P450c17 (c17) mRNA expression in cultured NCI-H295R cells. 28S rRNA was used as a loading control. The bars represent StAR/28S and P450c17/28S mRNA expression (means ± S.E.M.) in three 48 h experiments when control (0 µg/ml or ng/ml) is adjusted to 100%. Different letters above the bars indicate statistically significant differences (P < 0.05) between the treatments. Please note that the statistical significances have been estimated independently for each parameter; thus different parameters (StAR and P450c17) should not be compared with each other. A representative Northern blot hybridized with StAR, P450c17 and 28S ribosomal RNA probes is presented below the bar figure.

Figure 6 The effect of 8-BrcAMP (0.1 mM; cAMP), activin A (40 ng/ml; Act), inhibin A (40 ng/ml; Inh), and their combinations on cortisol, androstenedione, DHEA and DHEA-S secretion in cultured NCI-H295R cells. The bars represent relative steroid secretion (means ± S.E.M.) in four separate 48 h experiments with the control (C) adjusted to 100%. Different letters above the bars indicate statistically significant differences (P < 0.05) between the treatments. Please note that the statistical significances have been estimated independently for each parameter; thus different parameters (e.g. cortisol and DHEA) should not be compared with each other.
Activin A induced apoptotic changes in cultured NCI-H295R cells. This effect has also been shown in fetal adrenal cells and activins are therefore believed to contribute to the postnatal remodeling of the adrenal cortex (Spencer et al. 1999). Apoptosis is also known to occur in the adrenal cortex during the adult period. It has been assumed that adrenocortical cells proliferate in the outermost layers of the cortex and then migrate towards the inner cortex and die through an apoptotic mechanism in the ZR (reviewed in Wolkersdörfer & Bornstein 1998). The mechanisms controlling apoptosis in the adrenal cortex are complicated, but it is possible that activins play a role in this process as their signaling system is expressed in the same area as the most active apoptosis (Sasano et al. 1995). Interestingly, increased inhibin α-subunit expression has been detected in cortisol-producing and virilizing tumors probably originating from the inner cortical cells (Pelkey et al. 1998, Arola et al. 2000). Therefore, it is possible that decreased activin production or disturbed activin signal transduction in these inhibin α-subunit overexpressing cells could contribute to increased steroidogenesis and cell mass in these tumors.

In summary, we have shown that human adrenocortical cells express activin/inhibin receptors and intracellular signaling molecules suggesting activin/inhibin signaling potential especially in the ZR and the innermost ZF of the human adrenal cortex. Activin decreases StAR and P450c17 gene expression leading to decreased glucocorticoid and androgen secretion, and causes apoptotic cell death. Thus, the activin–inhibin system may regulate steroid production and apoptotic cell death in the inner layers of the adrenal cortex and may also be involved in the development of hormonally active tumors in these zones.

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![Graph](https://via.placeholder.com/150)

**Figure 8** Dose-dependent effect of activin A on the apoptotic index of cultured NCI-H295R cells. The cells were cultured in the presence of activin A (0–30 ng/ml) for 2 days, fixed with methanol, and the apoptotic nuclei were stained with the TdT labeling method. The bars represent apoptotic index (means ± S.E.M.; number of apoptotic nuclei in relation to the total number of nuclei, five separate experiments). *P < 0.01 comparing control vs treatment. Light microscopic figures below the bar figure represent the control (A) and activin A-treated (30 ng/ml) cells (B). Black spots represent positive staining for fragmented DNA typical of apoptosis. Nuclear Fast Red counterstain was used to label the nuclei.


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