

Aminoglutethimide suppresses adrenocorticotropin receptor expression in the NCI-h295 adrenocortical tumor cell line

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Abstract

The adrenostatic compound aminoglutethimide (AG), a potent inhibitor of the P450 side chain cleavage enzyme, is used in the treatment of ACTH-dependent or adrenal Cushing's syndrome. Recently, AG has been shown to inhibit ACTH receptor (ACTH-R) mRNA expression in ovine adrenocortical cells in a time-dependent fashion. To investigate whether ACTH-R down-regulation will also be induced in tumor cells, we studied the effect of AG on ACTH-R expression in the human NCI-h295 adrenocortical carcinoma cell line, which expresses functional ACTH receptors and produces steroids of the glucocorticoid, mineralocorticoid and androgen pathway. The cells were incubated in triplicate with increasing doses of AG (3, 30, 300 μ M) which suppressed steroid secretion dose-dependently. After 48 h, cells were harvested, and total RNA was extracted, electrophoresed, blotted and hybridized with a human ACTH-R cDNA probe. In parallel experiments, after preincubation with AG the cells were stimulated with ACTH (10 nM) for 10 min and the intracellular cAMP accumulation was determined by RIA. AG significantly suppressed the baseline ACTH-R mRNA expression in a dose-dependent fashion (300 μ M AG, $5 \pm 1\%$; 30 μ M AG, $64 \pm 1\%$; 3 μ M AG, $108 \pm 19\%$ compared with control cells, $100 \pm 11\%$). The reduced

ACTH-R mRNA expression was paralleled by low ACTH-induced cAMP accumulation indicating reduced expression of the ACTH-R protein. The adrenostatic compound metyrapone, an inhibitor of 11β -hydroxylase activity, also suppressed ACTH-R mRNA expression in a similar fashion. Stimulation of the protein kinase A pathway by simultaneous incubation of ACTH (10 nM) or forskolin (10 μ M) together with AG was not able to overcome the steroid biosynthesis blockade, but reversed the inhibitory effects of AG on the ACTH-R mRNA expression. Also, cortisol (12 μ M) reversed the AG-induced ACTH-R mRNA expression. We conclude that AG induces profound ACTH-R down-regulation in the NCI-h295 cell line either by affecting the gene expression or by decreasing transcript accumulation via an effect on RNA stability. This novel action of AG can be reversed by stimulation of the cAMP pathway and of the glucocorticoid-mediated signal transduction cascade. As the down-regulation occurs *in vitro* at concentrations which are reached during treatment with AG in humans it may contribute to its therapeutic activity in adrenal disease.

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Introduction

Adrenocorticotropin (ACTH) is the major hormone that regulates glucocorticoid secretion, adrenal androgen secretion and, together with angiotensin II, mineralocorticoid secretion of the adrenal cortex. ACTH acts through its specific receptor, the ACTH receptor (ACTH-R), a member of a superfamily of the seven transmembrane G-protein-coupled receptors (Mountjoy *et al.* 1992) which is expressed in all three zones of the adrenal cortex. Activation of the ACTH-R leads to stimulation of the cAMP protein kinase A pathway, but the activation of other signal transduction cascades by ACTH such as the

protein kinase C (Arola *et al.* 1994, Armelin *et al.* 1996, Parissenti *et al.* 1996) and the lipo-oxygenase pathway (Yamazaki *et al.* 1996) have also been described. The ACTH-R belongs to the melanocortin receptor family which consists of four melanocyte-secreting hormone (MSH) receptors (MC-R 1, 3 to 5) and the ACTH (or MC-R 2) receptor (Magenis *et al.* 1994). The ACTH-R is mainly expressed in the adrenal cortex, but has recently been identified in human skin (Slominski *et al.* 1996) and rodent adipocytes (Boston & Cone 1996).

The role of ACTH-R mutations and ACTH-R expression in adrenocortical tumorigenesis is still ill-defined. Constitutively activating point mutations, such as those

found in the thyrotropin (TSH) receptor gene in toxic thyroid adenomas, have not been identified in adrenocortical tumors (Latronico *et al.* 1995, Light *et al.* 1995). Studies investigating ACTH-R expression by Northern blot or *in situ* hybridization found up-regulated ACTH-R mRNA levels in functional adrenocortical adenomas, but low ACTH-R mRNA expression in non-functional adenomas (Reincke *et al.* 1997, 1998). In these experiments, ACTH-R mRNA expression correlated well with the expression of P450 side chain cleavage (P450 scc) enzyme expression suggesting regulation by similar, as yet unidentified factors. *In vitro*, stimulation of the protein kinase A pathway induced up-regulation of both ACTH-R mRNA (Mountjoy *et al.* 1994) and P450 enzymes (Staels *et al.* 1993) in the human adrenocortical carcinoma cell line NCI-h295.

The adrenostatic compound aminoglutethimide (AG) is used in the treatment of patients with ACTH-dependent or adrenal Cushing's syndrome. AG acts mainly by inhibition of P450 scc (Vanden Bossche 1992). In 1987, Darbeida and colleagues reported inhibition of the ACTH-induced cAMP production in ovine fasciculata-reticularis cells by AG (Darbeida & Durand 1987, Darbeida *et al.* 1987). This suggested that adrenostatic treatment with AG may modulate the ACTH-R expression in the adrenal cortex. This hypothesis was recently confirmed by the same group showing that the abundance of ACTH-R mRNA in ovine adrenocortical cells was decreased by 1000 μ M AG (Picard-Hagen *et al.* 1997). The effect of AG in this system was prevented by concomitant treatment with dexamethasone, demonstrating that AG presumably acts via suppression of glucocorticoid synthesis.

Modulation of ACTH-R expression may be desirable in patients with adrenocortical carcinomas in whom endogenous ACTH secretion may influence tumor growth via the ACTH-R G-protein protein kinase-A signaling cascade. Therefore, we investigated the effects of AG on the ACTH-R mRNA expression and the ACTH-induced intracellular cAMP accumulation in the NCI-h295 cell line which may serve as a cellular model for functional adrenocortical carcinomas.

Materials and Methods

Materials

AG, forskolin, cortisol, insulin, metyrapone, selenium, and transferrin were obtained from Sigma (Deisenhofen, Germany). RPMI 1640, fetal calf serum (FCS) and trypan blue stain were supplied by Life Technologies (Eggenstein, Germany). Synthetic ACTH₁₋₂₄ was purchased from Ciba-Geigy (Wehr, Germany). [α -³²P]dCTP was from Amersham Buchler (Braunschweig, Germany). Quickhyb and NucTrap purification columns were obtained from Stratagene (Heidelberg, Germany) and Qiabrane Nylon membrane from Qiagen (Hilden, Germany).

AG was dissolved in 65% ethanol as a stock solution. The final ethanol concentration in the experiments was <0.1%. Metyrapone was solubilized in 10 mM acetic acid (final concentration <20 μ M).

Cell culture

The NCI-h295 tumor cell line (Gazdar *et al.* 1990), provided by A F Gazdar (National Cancer Institute, NIH, USA), was maintained in RPMI 1640 medium supplemented with transferrin (0.1 mg/ml), insulin (5 μ g/ml), selenium (5.2 μ g/ml) and 2% FCS (=TIS medium). Cells were grown as suspension cells in 175 cm² flasks at 37 °C under an atmosphere of 5% CO₂-95% air. For the experiments, the cells were grown in 75 cm² flasks (approx. 1.0 million cells/ml). The cells were incubated for 48 h with AG (3, 30, 300 μ M), metyrapone (300 μ M), ACTH (10 nM), forskolin (10 μ M), AG (300 μ M) plus ACTH (10 nM), AG (300 μ M) plus forskolin (10 μ M), AG (300 μ M) plus cortisol (12 μ M) or metyrapone (300 μ M) plus forskolin (10 μ M). Each experiment was performed in triplicate. At the end of the incubation period the medium was removed by centrifugation and stored at -20 °C for subsequent hormone determination. Cells were examined by trypan blue staining for cell viability, counted with a coulter counter (CASY 1, Schärfe, Reutlingen, Germany), washed with PBS and prepared for RNA extraction. To determine the response of cAMP to ACTH stimulation the cells were grown in 24-well plates (1.5 million cells per well) in TIS medium. Cells were pretreated with and without AG (3-300 μ M) for 48 h. After washing with PBS, the cells were incubated with and without ACTH (10 nM) or forskolin (10 μ M) for 10 min.

Steroid determination and cyclic AMP analysis

Cortisol, aldosterone and dehydroepiandrosterone sulfate (DHEA-S) were determined in the supernatant from about 1.0 million cells/ml by radioimmunoassay (DPC Biermann, Bad Nauheim, Germany) according to the instructions of the manufacturer. Cyclic adenosine-3',5'-monophosphate was measured by means of an RIA (Amersham Buchler) on whole cell suspensions after ethanol extraction to determine the intracellular cAMP content.

RNA extraction and Northern analysis

Total RNA was extracted using the guanidinium isothiocyanate method (RNeasy kit, Qiagen). The RNA content was estimated by spectrophotometry. Twenty-five micrograms total RNA were denatured in 2.2 M formaldehyde and electrophoresed in a 1% agarose-formaldehyde gel. The integrity of the major RNA species was examined under UV light to ensure consistency between lanes. The RNA was blotted by capillary transfer onto a nylon

Table 1 Dose-dependent effect of AG on steroid biosynthesis and cell proliferation in the NCI-h295 cell line. Cells were incubated for 48 h with AG, ACTH, forskolin (FSK), hydrocortisone (F), metyrapone (MTP) or combinations of these compounds as indicated. The steroid concentrations in the supernatant and the cell number are expressed as a percentage of untreated control cells (=100%). Values are the mean \pm S.E.M. of 3 separate experiments. Control cells secreted 1075 ng cortisol/10⁶ cells/48 h, 95 ng DHEA-S/10⁶ cells/48 h and 5 ng aldosterone/10⁶ cells/48 h. Note that AG treatment did not influence cell growth whereas stimulation of the protein kinase A pathway with forskolin reduced cell proliferation of the NCI-h295 cell line

	Cortisol (% of control)	DHEA-S (% of control)	Aldosterone (% of control)	Cell number (% of control)
Control	100.0 \pm 4	100.0 \pm 9	100.0 \pm 2	100.0 \pm 8
AG (300 μ M)	1.3 \pm 1	8.2 \pm 1	6.1 \pm 1	96.9 \pm 6
AG (30 μ M)	23.7 \pm 1	40.6 \pm 3	23.6 \pm 3	106.9 \pm 8
AG (3 μ M)	90.2 \pm 1	95.0 \pm 6	91.1 \pm 2	91.5 \pm 2
MTP (300 μ M)	3.0 \pm 1	11.5 \pm 2	9.9 \pm 1	98.9 \pm 9
AG (300 μ M)+ACTH (10 nM)	1.3 \pm 1	8.8 \pm 2	8.8 \pm 1	93.2 \pm 7
AG (300 μ M)+FSK (10 μ M)	5.3 \pm 1	19.7 \pm 6	11.6 \pm 1	67.9 \pm 14
AG (300 μ M)+F (12 μ M)	—	17.2 \pm 1	12.8 \pm 1	88.5 \pm 8
MTP (300 μ M)+FSK (12 μ M)	5.1 \pm 1	28.2 \pm 2	15.1 \pm 2	71.1 \pm 4
ACTH (10 nM)	144.3 \pm 5	189.3 \pm 16	134.4 \pm 13	91.8 \pm 8
FSK (10 μ M)	418.0 \pm 16	627.2 \pm 95	305.2 \pm 32	69.6 \pm 6

membrane and cross-linked by exposure to UV radiation. Prehybridization was carried out at 68 °C with Quickhyb solution for 1 h. Hybridization was performed in the same buffer at 68 °C for 1 h using a 1061 base pair, PCR-generated fragment of the human ACTH-R probe (Reincke *et al.* 1997) labeled with [³²-P]dCTP (Random Primed DNA Labeling Kit, Boehringer Mannheim, Germany). Unincorporated nucleotides were separated from radiolabeled DNA probes by NucTrap purification columns. After washing at 60 °C twice for 15 min in 1 \times SSC and twice for 15 min in 0.5 \times SSC (each containing 0.1% SDS), the blots were exposed to a Kodak X-OMAT-DS film (Kodak, Fernwald, Germany) at -78 °C with intensifying screens.

The blots were stripped and rehybridized with a PCR-generated angiotensin II receptor type 1 cDNA (Nawata *et al.* 1995) and with a mouse β -actin cDNA probe for standardization (Stratagene). The relative intensity of the major hybridization signals (ACTH-R, 1.8 kb transcript; angiotensin II receptor type 1, 2.3 kb transcript) was quantified by scanning densitometry. Autoradiographic images were digitalized with a video camera and a Macintosh PowerMac 7100 computer-based image analysis system using the IMAGE program (NIMH, National Institutes of Health, Bethesda, MA, USA). The results were expressed as a percentage of the untreated control cells (=100%) after normalization for β -actin expression.

Statistical analysis

All results are expressed as means \pm S.E.M. Significances of differences were evaluated by ANOVA (Statview 4.51). A value of $P < 0.05$ was considered statistically significant.

Results

Treatment of NCI-h295 cells for 48 h with AG led to a dose-dependent decrease of the cortisol biosynthesis. The secretion of other steroids (DHEA-S and aldosterone) was suppressed in a similar manner (Table 1). Incubation with AG did not affect the cell viability which was greater than 90% by trypan blue inclusion in all experiments. In cell proliferation experiments control cells had a population doubling time of 70 h while cells treated with 300 μ M AG still doubled in 78 h. After 48 h there was no significant difference in cell number between the AG-treated groups and control cells (Table 1).

Incubation of the cells for 48 h with AG significantly reduced ACTH-R mRNA expression in a dose-dependent fashion while the angiotensin II receptor type 1 mRNA abundance was unaffected. In contrast, forskolin increased the accumulation of ACTH-R mRNA, decreased the expression of angiotensin II receptor type 1 mRNA, and decreased cell proliferation, as shown in part previously (Mountjoy *et al.* 1994, Bird *et al.* 1995b) (Fig. 1A,B, Table 1).

To investigate the functional significance of reduced ACTH-R mRNA levels, we evaluated the cAMP response of NCI-h295 cells to ACTH and forskolin. Forty-eight hours incubation with AG did not influence baseline cellular cAMP synthesis (300 μ M AG, 746 \pm 25; 30 μ M AG, 864 \pm 3; 3 μ M AG, 734 \pm 41 vs control cells, 796 \pm 21 fmol/well/10 min; P =not significant). However, ACTH-stimulated cAMP accumulation was significantly reduced after pretreatment with AG in doses of more than 30 μ M indicating reduced functional ACTH-R expression (Fig. 2). In parallel experiments,

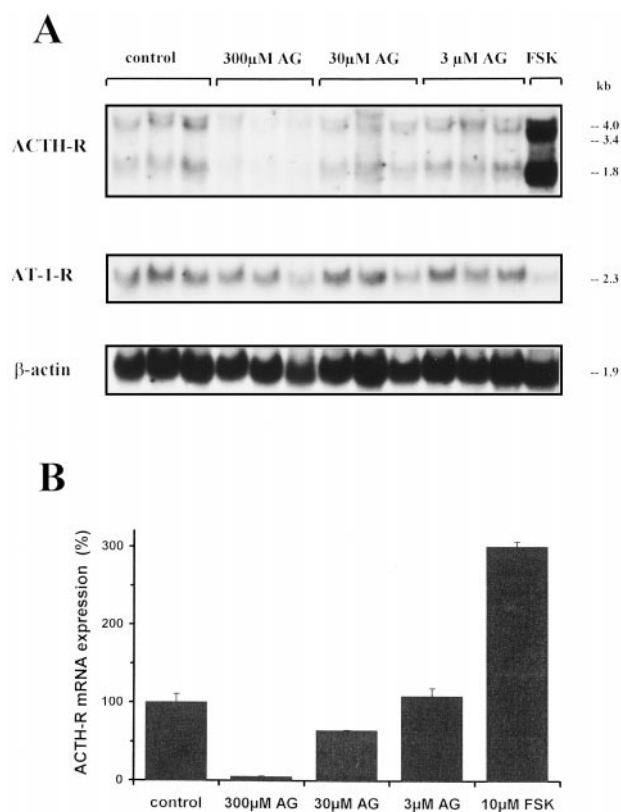


Figure 1 Effect of adrenostatic treatment with AG and forskolin (FSK) (10 μ M) on ACTH-R mRNA and angiotensin II receptor type 1 mRNA expression in the NCI-h295 cell line. (A) Northern blot of ACTH-R mRNA, angiotensin II receptor type 1 mRNA (AT-1-R) and β -actin mRNA expression. (B) Mean ACTH-R mRNA expression \pm S.E.M. of the 1.8 kb transcript as a percentage of control cells (100%) normalized for β -actin expression is shown. Results were significantly different between groups ($P < 0.01$).

after preincubation with 300 μ M AG, the forskolin-induced cAMP synthesis was still present (AG alone, 746 ± 35 vs AG/forskolin, 1082 ± 36 vs forskolin alone, 1254 ± 26 fmol/well/10 min) demonstrating that reduced responsiveness to ACTH was not due to diminished adenylyl cyclase activity.

Stimulation of the protein kinase A pathway by simultaneous incubation of ACTH (10 nM) or forskolin (10 μ M) together with AG was not able to overcome the steroid biosynthesis inhibition, but reversed the inhibitory effects of AG on the ACTH-R mRNA expression to baseline without restoring full response (Table 1 and Fig. 3). Similar effects on ACTH-R expression were seen with incubation of metyrapone with or without forskolin (Fig. 4). This demonstrates that cAMP is one of the components involved in ACTH-R regulation independent of steroid biosynthesis. On the other hand, incubation of cortisol (12 μ M) together with AG showed a significant up-regulation of the ACTH-R mRNA compared with

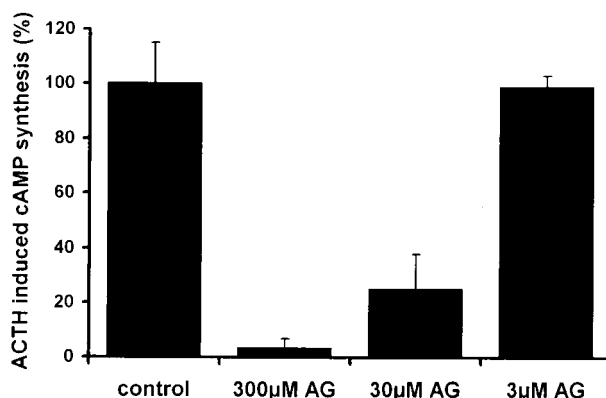


Figure 2 Effect of pretreatment with AG on the ACTH-induced cAMP response in the NCI-h295 cell line. Cells were pretreated in triplicate with or without AG (3–300 μ M) for 48 h. The mean cAMP synthesis \pm S.E.M. over baseline as a percentage of control cells (100%) is shown. There were significant differences between groups ($P < 0.01$). The ACTH-induced cAMP synthesis of control cells was 1130 ± 118 fmol/well/10 min.

cells incubated with AG only, indicating that glucocorticoids at higher concentrations may also influence ACTH-R expression (Fig. 3).

Discussion

Our study shows that incubation of the NCI-h295 cell line with AG suppresses the ACTH-R mRNA abundance in a dose-dependent fashion. This down-regulation could either be caused by decreased gene expression or by diminished transcript accumulation via reduced RNA stability. Decreased ACTH-R mRNA levels are paralleled by a profound reduction of the cAMP response to ACTH, demonstrating reduced expression of functional ACTH-R in this system. The actions of AG on ACTH-R mRNA expression in the NCI-h295 cell line can be reversed with both forskolin and cortisol suggesting that, in addition to the protein kinase A pathway, the glucocorticoid signal transduction cascade is involved in regulation of ACTH-R expression.

Our data confirm and extend the recent report of Picard-Hagen *et al.* (1997) on the effects of glucocorticoids and AG on ACTH-R mRNA expression in ovine adrenocortical cells. In their experiments, AG (1000 μ M) decreased ACTH-R mRNA expression, an effect which was prevented by concomitant treatment with dexamethasone. In addition, AG diminished the ACTH-R up-regulation which can be induced by stimulation with ACTH. Our data show that the AG-induced ACTH-R down-regulation is not only present in normal ovine adrenocortical cells but can also be demonstrated in a human adrenocortical tumor cell line. It can, therefore, be expected that AG modulates ACTH-R expression *in vivo*.

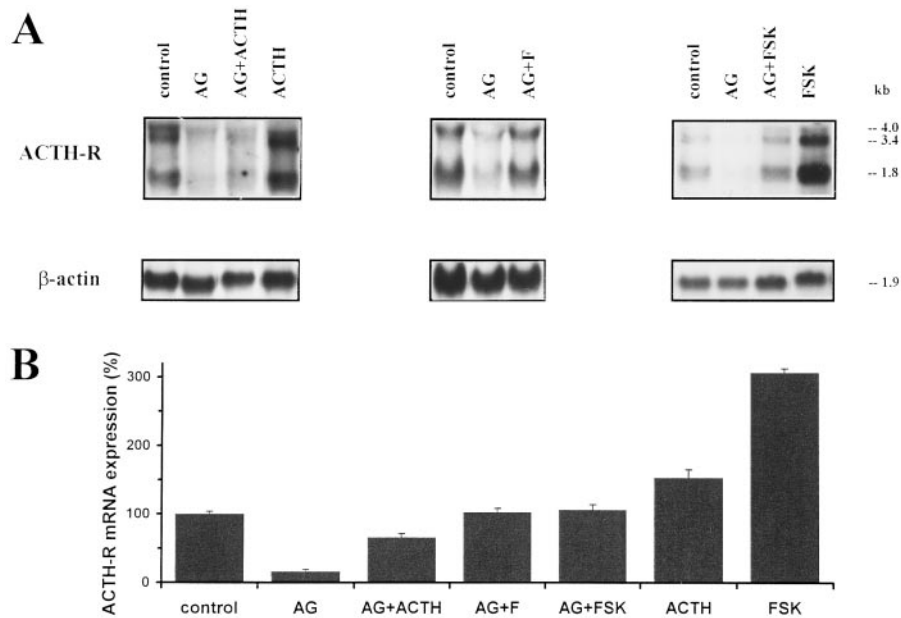


Figure 3 Effects of simultaneous incubation of 300 μ M AG with and without 10 nM ACTH, 12 μ M cortisol (F) or 10 μ M forskolin (FSK) on ACTH-R mRNA expression in the NCI-h295 cell. (A) Northern blot of ACTH-R mRNA expression (top) and β -actin mRNA expression (bottom) from 3 different experiments. (B) Mean ACTH-R mRNA expression \pm S.E.M. of the 1.8 kb transcript as a percentage of control (100%) normalized for β -actin expression is shown. Results were significantly different between groups ($P < 0.01$).

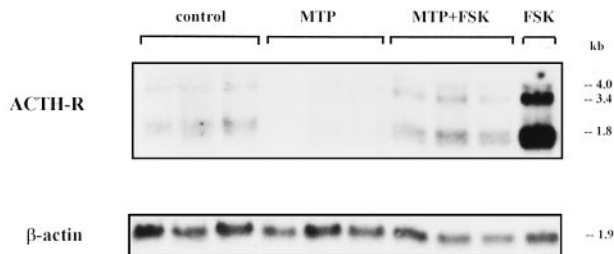


Figure 4 Effect of 300 μ M metyrapone (MTP) with or without 10 μ M forskolin (FSK) on ACTH-R mRNA expression in the NCI-h295 cells. The blot was hybridized with cDNAs of ACTH-R (top) and of β -actin (bottom).

Such an effect on ACTH-R number may be desirable, especially in patients with adrenal pathology. For example, in patients with non-secreting or virilizing adrenocortical carcinoma, ACTH may be one of the factors stimulating cell proliferation via the protein kinase A pathway or other second messengers. In a previous study we showed that 25% of adrenocortical carcinomas express ACTH-R levels similar to those of the normal adrenal cortex (Reincke *et al.* 1997). Down-regulation of ACTH-R expression in these tumors could desensitize the tumor cells to the stimulatory action of ACTH.

We used the NCI-h295 cell line to determine the effects of AG on ACTH-R expression. This cell line is

derived from a human functional adrenocortical carcinoma and has been maintained in cell culture since 1981. It displays a differentiated cellular phenotype with production of more than 30 different steroids of the mineralocorticoid, glucocorticoid and adrenal androgen pathways (Gazdar *et al.* 1990, Rainey *et al.* 1994). The coexpression of multiple P450 enzymes in cells of the NCI-h295 cell line resembles the steroidogenesis found in the fetal adrenal gland (Staels *et al.* 1993). In addition to P450 enzymes, the cell line expresses functional receptors for angiotensin II (Bird *et al.* 1993, 1994, 1995a), ACTH (Mountjoy *et al.* 1994) and insulin-like growth factor-I (Reincke *et al.* 1996). Regulation of ACTH-R expression in this cell line seems to be similar to that of the normal human adrenal cortex, although the magnitude of the responses to stimulation by ACTH is smaller because of a lower number of ACTH-Rs. Whereas the NCI-h295 cell line – with some limitations – may serve as a model of the functional neoplastic adrenal cortex, other tumor cell lines are less suitable for our purposes: in the case of the human SW-13 cell line because of missing ACTH-R expression, in the case of the mouse Y-1 cell line because of the lack of ability to synthesize cortisol due to missing 17 α -hydroxylase expression.

The observed suppression of ACTH-R expression in the NCI-h295 cell line seems to be a specific and substance-related effect: AG was used in a dosage within the 'therapeutic range' (Meyer 1994), in contrast to other

studies which employed higher concentrations up to 1000 μM (Darbeida & Durand 1987, Urban *et al.* 1990, Picard-Hagen *et al.* 1997). AG acts by inhibition of P450 scc and aromatase activity with an IC_{50} of 16–40 μM (Vanden Bossche 1992). The plasma levels in patients treated with 1000 mg AG/day reach 70 $\mu\text{mol/l}$ (Strocchi *et al.* 1991). In our system, AG in concentrations of 300 μM did not significantly influence cell viability and cell number, thus arguing against a toxic effect of this compound. This is also supported by the unchanged mRNA abundance of the angiotensin-II receptor type 1. Therefore, we believe that modulation of ACTH-R expression is a new and substance-related pharmacological effect of AG. The mechanisms involved appear to be cAMP-independent, since basal cAMP production was not changed by AG. We also observed down-regulation of ACTH-R mRNA abundance by metyrapone, an inhibitor of P450 11 β -hydroxylase which converts 11-deoxycortisol to cortisol. Similar to AG, metyrapone was used in a dosage within the 'therapeutic range' (Jubiz *et al.* 1970) which did not influence cell viability. This suggests that the ACTH-R modulation is not restricted to AG but may be a general effect of this class of substances.

The expression of ACTH receptor mRNA and ACTH binding sites of normal human and bovine adrenocortical cells is up-regulated 1.2- to 20-fold by exposure to ACTH (Durand & Locatelli 1980, Penhoat *et al.* 1989, Saez *et al.* 1989, Rainey *et al.* 1991, Penhoat *et al.* 1993, 1994, Lebrethon *et al.* 1994a,b, Morita *et al.* 1995, Penhoat *et al.* 1995). This effect is mediated through the cAMP signal transduction cascade since it can be mimicked by treatment with forskolin and cAMP. Accordingly, several putative cAMP response elements have been identified in the promoter of the human ACTH-R gene (Naville *et al.* 1996), suggesting direct stimulation of ACTH-R transcription by cAMP. In addition, up-regulation of ACTH-R expression by angiotensin II through non-protein kinase A pathways has been reported (Mountjoy *et al.* 1994, Lebrethon *et al.* 1994b, Penhoat *et al.* 1994). *In vivo*, down-regulation of ACTH-R mRNA has been shown in rats treated with dexamethasone i.p. suppressing plasma ACTH levels (Morita *et al.* 1995). Similarly, ACTH-R mRNA expression was low in a knock-out mouse model in which the pro-opiomelanocortin promoter was deleted and consecutively low plasma ACTH concentrations were obtained (Allen *et al.* 1995). ACTH-R expression in neoplastic adrenal tissue seems to be regulated by additional factors other than plasma ACTH, since high ACTH-R mRNA levels have been found in adrenal adenomas of patients with adrenal Cushing's syndrome and suppressed plasma ACTH (Morita *et al.* 1995, Reincke *et al.* 1997, 1998).

It is noteworthy that concomitant incubation of AG with forskolin reversed ACTH-R expression to baseline without giving full response. This supports the concept that AG may act through more than one pathway or it can

be explained by an AG-inducible suppressor of ACTH-R transcription. Recently, in the mouse ACTH-R gene promoter, binding of a transcriptional repressor or silencer has been identified between position –1236 bp and –908 bp (Cammass *et al.* 1997). It has been postulated that this factor is responsible for tissue-specific expression of the ACTH-R in ACTH target tissues. Induction of such a repressor by AG in our system could explain why the forskolin effect on ACTH-R expression remained impaired during AG treatment. However, other potential mechanisms of action of AG may involve interaction with the steroidogenic factor 1 (SF-1), an orphan member of the nuclear receptor superfamily (Lala *et al.* 1992) or with its potential oxysterol ligands (Lala *et al.* 1997). SF-1 binding sites have been identified in the mouse and human ACTH-R promoter (Naville *et al.* 1996), and transfection studies showed enhanced ACTH-R transcription if the SF-1 coding region was co-transfected with the mouse ACTH-R promoter into fibroblast L-cells (Cammass *et al.* 1997).

Apparently an important factor for the observed ACTH-R down-regulation is inhibition of glucocorticoid production by AG. Glucocorticoids enhance the ACTH-induced cAMP or cortisol response in adrenal cells or guinea pig adrenal glands, probably via increased ACTH-R expression (Darbeida & Durand 1987, 1988, 1990, Mokuda *et al.* 1997). Recently, Picard-Hagen *et al.* (1997) demonstrated convincingly that glucocorticoids stimulate corticotropin receptor mRNA levels in a time- and dose-dependent manner. Dexamethasone at 10^{-6} M induced a mean increase of $144 \pm 11\%$ over control. This enhancing effect was specific for glucocorticoids since the anti glucocorticoid RU486 blocked the effect of dexamethasone, whereas other steroids such as testosterone did not modify ACTH-R mRNA levels. In our experiments using the physiological glucocorticoid, cortisol, concentrations of 12 $\mu\text{mol/l}$ (equivalent to the cortisol concentrations achieved by forskolin stimulation in our system) induced a significant ACTH-R mRNA up-regulation counteracting the effects of AG. This glucocorticoid-mediated effect on ACTH-R expression suggests an ultra-short positive intra-adrenal feedback mechanism within the zona fasciculata by which increased glucocorticoid production sensitizes zona fasciculata cells to the stimulatory action of ACTH. However, a glucocorticoid response element has been identified only in the mouse, but not in the human ACTH-R gene promoter (Naville *et al.* 1996, Cammass *et al.* 1997).

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