REVIEW

ACTH-receptor expression, regulation and role in adrenocortical tumor formation

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Introduction

The production of adrenal glucocorticoids and androgens from the zona fasciculata and reticularis is predominantly regulated by adrenocorticotropin (ACTH) (1). Moreover, this 39-amino acid peptide derived from pituitary proopiomelanocortin contributes to the short-term secretion of mineralocorticoids from the zona glomerulosa (2). ACTH acts via a seven-transmembrane domain receptor belonging to the G-protein-coupled receptor superfamily leading to activation of the adenylate cyclase pathway and consecutive activation of protein kinase A (3). Activation of other signal-transduction cascades by ACTH, such as the protein kinase C (4–6) and the lipoxygenase pathway (7), has also been described. After long-term stimulation, ACTH increases the output of adrenal fasciculata cells by increasing the expression of several key steroidogenic enzymes (8). Additionally, ACTH is crucial for the development of the adrenal cortex and may play an essential part in adaptational processes like adrenal hypertrophy (9). The ACTH receptor is mainly expressed in the adrenal cortex, but has been identified in human skin (10), in an ovarian steroid cell tumor (11) and in rodent adipocytes (12). There is no evidence of ACTH-receptor expression in other tissues, implying that an effective mechanism exists to restrict expression of this gene.

Genomic organization

Substantial progress in the understanding of the expression and regulation of the ACTH receptor has been made since the cloning of the ACTH-receptor gene by the group of Cone in 1992 (13). The coding sequence of the human ACTH receptor contains no intron, but the presence of one intron (of about 18 kb) separating the coding exon 2 from an upstream untranslated exon 1 has been demonstrated. The major transcription start site is located in the second exon. Northern blot analysis of cultured human adrenocortical cells revealed several transcripts that can be partly explained by the use of different initiation sites of transcription (14). The genomic organization of the ACTH receptor is shown in Fig. 1. One kilobase of the promoter region of the ACTH receptor has been

cloned and the presence of several putative binding sites for transcriptional factors has been reported (15) (Fig. 2).

The ACTH receptor is a member of the superfamily of G-protein-coupled receptors with seven transmembrane domains (16). Together with several melanotropin receptors, the ACTH receptor belongs to the melanocortin receptor subfamily. Those receptors are characterized by short NH₂-terminal extracellular domains, short intracellular COOH-terminal domains as well as short fourth and fifth transmembrane-spanning domains (16). Up to now, the ACTH receptor is the shortest known G-protein-coupled receptor, consisting of 297 residues with a predicted molecular mass of 33 kDa. Two putative glycosylation sites are part of the NH₂-terminus of the ACTH receptor. Thus, glycosylation may account for the molecular mass of 43 kDa, which has been inferred by cross-linking experiments (17). Since no antibody against the human ACTH receptor is available to date, detailed experiments at the protein level are still hampered.

ACTH-receptor promoter and regulation of expression

Already prior to cloning of the ACTH receptor it has been demonstrated that the expression of ACTH-binding sites on human adrenocortical cells is up-regulated after exposure of the cells to ACTH (18). This unusual up-regulation by its own ligand was confirmed in recent studies showing up-regulation of ACTH-receptor mRNA and receptor-binding sites in human adrenocortical cells after stimulation with ACTH (19). In addition, up-regulation of ACTH-receptor expression by angiotensin II has been reported, probably through pathways not involving protein kinase A (19, 20). ACTH not only increases the transcriptional rate of ACTH-receptor message but also prolongs the ACTH-receptor mRNA half-life (21). In addition, the number of ACTH-binding sites is also up-regulated by other activators of the cAMP pathway like dbcAMP and forskolin (20). These findings could explain why adrenal responsiveness to ACTH is impaired rapidly after suppression of endogenous ACTH and restored within hours in patients with secondary adrenal insufficiency exposed to i.v. administered exogenous

Figure 1 Diagrammatic representation of the proposed genomic structure of the ACTH-receptor gene and the mature mRNA transcript and protein. PstI indicates restriction sites for PstI-restriction polymorphism; CA repeat indicates the interval between 12 kb SacI and 9.5 kb BamHI fragment, where the highly polymorphic marker within the intron of the ACTH receptor is located. Adapted from (15) and (58). AS, amino acids.

ACTH (22). The effects of ACTH on ACTH-receptor expression are mediated through the cAMP signal-transduction pathway because they can be mimicked by treatment with forskolin and cAMP itself. Accordingly, several putative cAMP-responding elements have been identified in the promoter of the human ACTH-receptor gene, suggesting direct stimulation of ACTH-receptor gene transcription by cAMP (15). By deletion analysis the region between -764 and -503 of the promoter was shown to confer cAMP responsiveness, which was abolished after mutation of an AP-1 site (23). This indicates that cAMP responsiveness of the human ACTH-receptor promoter is mediated by AP-1 (Fig. 2).

Steroidogenic factor 1 (SF-1) is an orphan nuclear receptor which regulates several genes involved in

steroidogenesis (24) and is a development regulator critical for the formation of adrenals and gonads (25). SF-1 seems to play a pivotal role in determining the tissue-specific expression of its target genes. The presence of three SF-1-binding sites has been demonstrated in the ACTH-receptor promoter at position -35 , -98 and -209 bp (26–28). The SF-1 element at -35 bp is reported to be involved in the basal promoter activity but is not sufficient for the cAMP-regulated expression of the ACTH-receptor gene (29). Mutation in all three binding sites led to a marked decrease of promoter activity. Only the SF-1 elements at position -35 bp and -98 bp but not the -209 bp element seem to be major contributors to the forskolin-stimulated promoter activity (26).

Figure 2 Structure of the ACTH-receptor promoter region. Relative position of putative regulatory elements are shown. For details see text.

Figure 3 Localization of ACTH-receptor mRNA in human adrenal cortex by *in situ* hybridization. Upper left: bright-field microscopy ($\times 50$). Upper right: dark-field microscopy ($\times 50$) shows the presence of specific mRNA indicated by white staining. Intense mRNA accumulation is present in the zona glomerulosa (G), the zona fasciculata (F) and the zona reticularis (R), but not in the capsule (C) and the medulla (M) of the adrenal gland. Lower left: P450 17-hydroxylase mRNA expression of the same adrenal slice with absent mRNA in the zona glomerulosa and high mRNA expression in the zona fasciculata and in the zona reticularis. Lower right: control section hybridized with the ACTH-receptor cDNA after RNase pretreatment. Magnification $\times 50$. Reproduced from (41) by permission of the Journal of Endocrinology Ltd.

Conversely, ACTH-receptor expression can be suppressed by adrenal-specific transcription factors, like DAX-1 (dosage-sensitive sex reversal adrenal hypoplasia congenita critical region on the X chromosome gene 1). DAX-1 is a repressor of several P450 enzymes in the adrenal gland and has been shown to act by multiple mechanisms (30). We investigated ACTH-receptor gene regulation by DAX-1 using the mouse Y1 tumor cell line transfected with the human ACTH-receptor promoter coupled to a luciferase reporter gene. Co-expression of DAX-1 clearly reduced basal and forskolin-stimulated gene expression. The effect of DAX-1 appears to be mediated by interaction with SF-1, as elimination of the SF-1 binding site in the ACTH-receptor promoter by site-directed mutagenesis abolished its suppressive action (27). Further evidence for a suppressive effect of DAX-1 comes from adrenocortical tumor tissues: DAX-1 and ACTH-receptor mRNAs show a negative correlation in adrenocortical tumors supporting an *in vivo* effect of DAX-1 on the ACTH-receptor promoter (31).

It has been reported that glucocorticoids enhance the ACTH-induced cAMP or cortisol response in adrenal cells or guinea pig adrenal glands (32–34). This effect was hypothesized to be mediated by up-regulation of ACTH-receptor expression by glucocorticoids. Recently, stimulation of ACTH-receptor mRNA by glucocorticoids has been demonstrated in a time- and dose-dependent manner (35). This enhancing effect was specific for glucocorticoids because the anti-glucocorticoid RU486 blocked the effect of dexamethasone, whereas other steroids such as testosterone did not modify ACTH-receptor mRNA levels.

Conversely, down-regulation of ACTH-receptor mRNA *in vivo* has been shown in rats treated with i.p. dexamethasone leading to suppressed plasma ACTH levels (36). Similarly, ACTH-receptor mRNA expression was low in a knockout mouse model with a deleted proopiomelanocortin promoter, which led to low plasma ACTH levels (37). Treatment of the human adrenocortical tumor cell line NCI-h295 with the adrenostatic compounds aminoglutethimide and

Figure 4 *In situ* hybridization of the ACTH-receptor (ACTH-R) mRNA, P450 side chain cleavage enzyme (P450scc) mRNA, and P450 17-hydroxylase (P450c17) mRNA in an aldosterone-producing adenoma. Control sections (+RNase) were pretreated with RNase before incubation with the ACTH-receptor cDNA. The adjacent normal adrenal cortex is visible on the right (arrow). Magnification $\times 5$. Reproduced from (41) by permission of the Journal of Endocrinology Ltd.

metyrapone led to down-regulation of ACTH-receptor mRNA (35, 38). This effect can be reversed by stimulation of the cAMP pathway and of the glucocorticoid-mediated signal-transduction cascade.

***In vivo* ACTH-receptor expression**

The adrenal cortex is composed of three distinct zones with different morphological and functional characteristics: the zona glomerulosa, which secretes mainly mineralocorticoids, the glucocorticoid-producing zona fasciculata, and the innermost zona reticularis, which is the source of adrenal androgens. The physiology of steroid secretion suggests expression of the ACTH receptor in all three zones, since acute administration of ACTH leads to enhanced secretion of mineralocorticoids,

glucocorticoids and androgens (39, 40). In accord with this notion, ACTH-receptor mRNA was detected in all three adrenocortical zones by means of *in situ* hybridization (41) (Fig. 3). In mice, ACTH-receptor mRNA expression was found mainly in the zona glomerulosa and the zona fasciculata, whereas cells of the zona reticularis expressed ACTH-receptor mRNA to a lesser degree (42). Similar distribution of ACTH-receptor mRNA was found in normal bovine adrenal cortex with a more abundant expression in zona glomerulosa than in zona fasciculata and reticularis cells (43).

Possible involvement of ACTH-receptor expression in adrenocortical development was underlined in recent studies of *in situ* hybridization experiments in human (44) and baboon fetuses (45). In human fetal adrenal

tissue from midgestation, mRNA encoding the ACTH receptor was localized in cells from all cortical zones, with higher abundance in the definite zone (which corresponds to the zona glomerulosa) compared with the fetal zone (reflecting the zona reticularis). In the baboon fetal adrenal gland, a biphasic developmental expression of ACTH receptor was reported with the lowest expression in early gestation, high expression in midgestation and a decline of ACTH-receptor mRNA in late gestation respectively. Because the fetal adrenal is comprised mainly of the fetal cortical zone throughout gestation, the decrease in ACTH-receptor expression between mid- and late gestation seems to occur primarily in the latter zone and may signal a selective decline in tropic responsiveness of androgen biosynthesis within the baboon fetal adrenal gland.

ACTH-receptor localization and expression in adrenal tumors and cell lines

ACTH-receptor mRNA expression in tumor tissue

Aldosterone- and glucocorticoid-producing adrenocortical adenomas are responsive to ACTH *in vivo* and *in vitro*, suggesting the expression of functional ACTH receptors (46, 47). In accord with this notion, we found high ACTH-receptor mRNA expression using Northern blotting and *in situ* hybridization in cortisol-producing and aldosterone-producing adenomas, whereas non-functional adenomas and carcinomas had low or absent ACTH-receptor mRNA levels (31) (Fig. 4). An overexpression of ACTH-receptor mRNA in aldosterone-producing adenomas was confirmed by findings of Arnaldi *et al.* (48). This finding supports the role of ACTH on aldosterone secretion as suggested by the presence of a diurnal rhythm. In a case of virilizing adrenocortical adenoma, ACTH-receptor mRNA was not detected and ACTH did not increase the *in vitro* production of androgens (49). Taken together, plasma ACTH concentrations seem to have no major influence on ACTH-receptor mRNA in hyperplastic and neoplastic adrenal tissue because ACTH mRNA levels were neither increased in adrenal hyperplasia due to Cushing's disease nor reduced in adrenal Cushing's syndrome with suppressed plasma ACTH.

ACTH-receptor modulation by adrenostatic compounds

Modulation of ACTH-receptor expression may be desirable in patients with adrenal pathology. The adrenostatic compound aminoglutethimide, a potent inhibitor of the P450 side chain cleavage enzyme, has been shown to inhibit ACTH-receptor mRNA expression in ovine adrenocortical cells in a time-dependent

fashion (33). We recently investigated whether aminoglutethimide suppresses ACTH-receptor expression in the NCI-h295 adrenocortical carcinoma cell line, which expresses functional ACTH receptors and produces steroids of the glucocorticoid, mineralocorticoid and androgen pathways (38). Aminoglutethimide significantly suppressed baseline ACTH-receptor mRNA expression in a dose-dependent fashion. This was paralleled by low ACTH-induced cAMP accumulation, indicating reduced expression of ACTH-receptor protein. The adrenostatic compound metyrapone, an inhibitor of 11 β -hydroxylase activity, also suppressed ACTH-receptor mRNA expression in a similar manner. These data show that aminoglutethimide and metyrapone induce profound ACTH-receptor down-regulation in the NCI-h295 cell line by as yet undefined mechanisms. Because the down-regulation occurs *in vitro* at concentrations that are reached during treatment with aminoglutethimide *in vivo* it may contribute to the therapeutic activity of adrenostatic compounds in adrenal disease.

ACTH-receptor mutations in adrenal tumors

No evidence for activating point mutations

cAMP is a key second messenger involved in hormone hypersecretion and/or increased cell proliferation in many endocrine tissues. Activating mutations of cAMP-regulating proteins, such as G-protein-coupled receptors and GTP-binding proteins have been implicated in a variety of human disorders including acromegaly and toxic thyroid adenomas (50, 51). Oncogenic transformation of the thyrotropin-receptor gene by point mutations was found in approximately 30% of hyperfunctioning thyroid adenomas (51). The mutant receptors conferred constitutive activation of adenylate cyclase after *in vitro* transfection experiments. An alternative pathway increasing intracellular cAMP levels also associated with tumorigenesis is constitutive activating mutations in the α -chain of the stimulatory G-protein (Gs) found in growth hormone-producing adenomas and thyroid adenomas (51).

Adrenocortical tumorigenesis differs from pituitary and thyroid tumorigenesis because activation of the cAMP/protein kinase A pathway seems to be of little importance in the development of adrenocortical neoplasms. Although ACTH stimulates adrenal synthesis of steroidogenic enzymes, leading to increased steroid production and an increase in adrenal weight, a proliferative activity of ACTH has been questioned. Physiological ACTH levels are unable to induce compensatory adrenal hyperplasia after unilateral adrenalectomy in hypophysectomized animals (52). *In vitro*, inhibition of adrenal cell proliferation by physiological ACTH concentrations has been reported, and

even pharmacological doses of ACTH induce only moderate cell growth (53). In accord with this findings, activating point mutations of neither the ACTH receptor nor the α -chain of Gs have been identified in benign or malignant adrenocortical tumors (50, 54–56). On the contrary, activating mutations of the Gi2, one of the adenylyl cyclase inhibitory G-proteins, were found in a minority of adrenocortical tumors (50, 56, 57).

These data suggest that, in the adrenal cortex, the ACTH/G-protein kinase A signaling pathway is preferentially important for regulation of steroid hormone secretion and, hence, maintenance of a highly differentiated cellular phenotype while it seems to be of low importance for cellular proliferation.

Loss of constitutive heterozygosity (LOH) of the ACTH-receptor gene locus

Beside point mutations, loss or impairment of function of the ACTH-receptor gene could be caused by deletion of the ACTH-receptor gene locus. Recently, we identified a PstI polymorphism in the promoter region of the ACTH-receptor gene (58) (Fig. 1). The rate of heterozygosity for this polymorphism was 53.3%. In a series of 20 cases with benign and malignant adrenocortical tumors, LOH of the ACTH-receptor gene was associated with an advanced tumor stage and a more rapid course than in carcinoma patients without LOH. Northern blot experiments demonstrated reduced expression of ACTH-receptor mRNA in the tumors with LOH of the ACTH-receptor gene, suggesting functional significance of the finding at the transcriptional level.

In a recent approach, we discovered a highly polymorphic marker lying 9.5 kb to 6.0 kb upstream of the ACTH receptor coding exon 2, within the intron of the ACTH-receptor gene (Fig. 1). Using this novel microsatellite marker with 17 CA repeats a rate of heterozygosity of >98% could be obtained in 66 investigated individuals. LOH of the ACTH-receptor gene locus was found in 5 out of 13 carcinoma patients (38%), but in none of 30 adrenal adenomas, 9 pheochromocytomas or 14 other adrenal masses (59).

These data suggest that the ACTH receptor may act as a tumor-suppressor gene. Allelic loss of the ACTH-receptor gene in adrenocortical tumors can result in loss of differentiation, a characteristic feature of human tumorigenesis that is associated with clonal expansion of a malignant cell clone.

Summary

The regulation of the ACTH-receptor gene is unique in that it is up-regulated by its own ligand, ACTH. Ligand-induced up-regulation of ACTH-receptor expression may be an important adaptive process directed towards optimizing adrenal responsiveness to ACTH in the context of physiological stress and the maintenance of metabolic homeostasis in which the adrenals play a

pivotal role. Whereas enhancement by ligand-induced up-regulation permits a more efficient and rapid glucocorticoid response, negative feedback regulation of glucocorticoids in the hypothalamus and pituitary inhibits ACTH secretion and allows a balanced adrenal response to stress. Since the cloning of the promoter region of the ACTH receptor, considerable progress in the understanding of the regulatory processes has been made. The effects of ACTH on ACTH-receptor expression is dependent on cAMP, probably mediated through AP-1. The profound effect of three SF-1-binding sites in the ACTH-receptor promoter was demonstrated by deletion experiments. Conversely, ACTH-receptor expression can be suppressed by adrenal-specific transcription factors, like DAX-1.

Despite an extensive search, no activating ACTH-receptor mutations have been found in adrenal tumors, excluding the ACTH receptor as a relevant oncogene in adrenal tumorigenesis. However, the ACTH receptor may act as a differentiation factor as suggested by LOH in adrenal carcinomas with an undifferentiated tumor type. In benign adrenal tumors, a strong correlation between ACTH-receptor expression and expression of P450 steroidogenic enzymes is evident. This close regulative relationship is lost in adrenal carcinoma, probably as a result of tumor dedifferentiation. Down-regulation of ACTH-receptor expression in normal and neoplastic tissue can be achieved by adrenostatic compounds such as aminoglutethimide and metyrapone.

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