EXPERIMENTAL STUDY

Steroidogenic acute regulatory protein mRNA expression in adrenal tumours

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Abstract

The rate limiting step in steroidogenesis is cholesterol transport through the outer to the inner mitochondrial membrane and the cytochrome P450 side chain cleavage (P450_{sc}) complex. The protein factor responsible for this transport, and as such necessary for regulating the acute production of steroids, has been identified and named the steroidogenic acute regulatory protein (StAR). We investigated the expression of StAR in functional and non-functional adrenal neoplasms and compared the expression with that of P450_{sc}. Poly A RNA was extracted from normal adrenal glands (NAG, $n = 5$), aldosterone producing adenomas (APA, $n = 4$), cortisol producing adenomas (CPA, $n = 5$), adrenocortical carcinomas (ACC, $n = 6$) and non-functional adenomas (NFA, $n = 3$), electrophoresed through a 1% agarose gel, blotted and hybridised with a PCR-generated cDNA labelled with [³²P]CTP. The blots were stripped and re-hybridised with a P450_{sc} cDNA and a mouse β -actin probe. Compared with P450_{sc}, StAR mRNA expression showed little variability in the magnitude of expression and did not correlate with the endocrine profiles (NAG: StAR $100 \pm 16\%$, P450_{sc} $100 \pm 14\%$; APA: StAR $80 \pm 3\%$, P450_{sc} $94 \pm 13\%$; CPA: StAR $71 \pm 10\%$, P450_{sc} $109 \pm 15\%$; NFA: StAR $64 \pm 9.5\%$, P450_{sc} $18 \pm 5\%$; means $\pm$ S.E.M.). ACC expressed low levels of both genes probably as a result of dedifferentiation (StAR $29 \pm 9\%$, P450_{sc} $46 \pm 18\%$). Incubation of the NCI-h295 tumour cell line with 10 nmol ACTH and 10 μ mol forskolin induced an increase in the abundance of StAR and P450_{sc} mRNA, demonstrating gene regulation by the cAMP protein kinase A pathway. Furthermore, we incubated the NCI-h295 tumour cell line with the adrenostatic compounds, aminoglutethimide and metyrapone. We could not detect an effect on the expression of StAR mRNA, whereas the expression of P450_{sc} mRNA was significantly reduced. We conclude that, in contrast to P450_{sc}, StAR seems to be evenly expressed in adrenocortical adenomas. Therefore, the endocrine activity of a given tumour cannot be explained by the abundance of StAR expression. In ACC, both StAR and P450_{sc} expression is low, explaining the relatively inefficient steroid production of these tumours.

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Introduction

Cholesterol is the basic substance for all steroid hormones. It is stored in intracellular lipid droplets. For acute steroid biosynthesis, cholesterol has to be mobilised and delivered from these lipid droplets to the P450 side chain cleavage (P450_{sc}) complex, which is associated with the inner mitochondrial membrane. This is achieved by transport of cholesterol through the outer to the inner mitochondrial membrane. This step is based upon the *de novo* synthesis of a cycloheximid sensitive protein (1–4). The respective protein was first purified by Clark *et al.* from MA-10 mouse Leydig tumour cells and was called steroidogenic acute regulatory protein (StAR) (5). The definitive proof of the role of StAR in steroid biosynthesis was shown by Lin *et al.* (6). They showed that a mutation in

the StAR gene causes congenital lipid adrenal hyperplasia, a severe disease that is characterised by impaired synthesis of all adrenal and gonadal steroid hormones (7–9).

The structural StAR gene is encoded on chromosome 8p11.2 (2, 10). StAR is synthesised as a 37 kDa precursor protein with an N-terminal mitochondrial target sequence, and is transformed into a 30 kDa protein during its transport into the mitochondria (2, 5, 11–13). The definite mechanism of the acute regulation of steroidogenesis by StAR is not known yet, but there are two proposed mechanisms: the association of StAR with the outer mitochondrial membrane facilitates the import of cholesterol, or the import of StAR to the inner mitochondrial membrane is concomitant with cholesterol transport. In both cases, the 37 kDa precursor protein would be the active form and

Table 1 Clinical data of the patients studied.

Tissue	No.	Age (years)	Sex	Tumour size (cm)
Normal adrenal glands of brain-dead patients	5	16–69	2F/3M	
Aldosterone producing adenoma	4	46–62	2F/2M	1.7–5.0
Cortisol producing adenoma	5	31–49	5F	1.8–7.0
Non-functional adenoma	3	35–68	3F	3.0–7.0
Adrenocortical carcinoma	6	1–79	4F/2M	8.0–12.0

F = female, M = male.

the cleavage of StAR to the 30 kDa form would mean the inactivation of that protein (2, 14).

Because StAR represents the rate limiting event in acute steroidogenesis, StAR gene expression could be one of the factors influencing steroidogenesis in adrenocortical neoplasms. Recently, Liu *et al.* reported low StAR expression in non-functional tumours and high levels of gene expression in functional adrenocortical tumours (15). This is in contrast to our data presented here that StAR seems to be constitutively expressed in these tumours.

Materials and methods

Tissues

Adrenal tissue from 18 patients with adrenal disease and from 5 non-neoplastic adrenal glands was studied. Clinical data of these patients are shown in Table 1. The clinical and pathological diagnosis was made according to established criteria (16–18). Non-functional adenomas were found incidentally by computed tomography performed for unrelated reasons. These patients were asymptomatic, and endocrine function tests were normal (urinary free cortisol, serum cortisol after 1 mg dexamethasone at 2300 h, plasma renin, serum aldosterone). Non-neoplastic adult adrenals ($n = 5$) were obtained after organs were removed from brain-dead patients for transplantation. Adrenal tumour tissue was collected with the approval of the ethical committee of the University Hospital of Würzburg. We investigated aldosterone-producing adenomas (APA, $n = 4$), cortisol producing adenomas (CPA, $n = 5$), non-functional adenomas (NEA, $n = 3$) and adrenocortical carcinomas (ACC, $n = 6$).

Only central parts of tumours were used, avoiding

necrotic areas and contamination with normal adjacent tissue. In addition, the integrity of the tissue was checked by light microscopy. The normal or neoplastic adrenal cortex was carefully dissected from the medulla with a scalpel using only the adrenal cortex for these experiments. The tissues were snap frozen and immediately stored at -78°C until analysed.

cDNA synthesis and purification of the StAR cDNA using reverse transcription-polymerase chain reaction (RT-PCR)

Poly A mRNA of a normal adrenal gland was reverse transcribed to first strand complementary DNA (cDNA) by the primers shown in Table 2 (6) using the GeneAmp Reverse Transcriptase RNA PCR Kit (Perkin Elmer Cetus, Norwalk, CT, USA). For the reverse transcription, 40 ng mRNA, 2 μl 10 $\times$ RTth-buffer, 2 μl 10 mmol MnCl_2 and 1.6 μl 10 mmol NTPs, and 10 pmol of the antisense primer, AS2, were incubated with 2 μl RTth polymerase (Perkin Elmer Cetus) for 15 min at 70°C . For the polymerase chain reaction, the reaction mix consisted of 64 μl H_2O , 8 μl 10 $\times$ chelating buffer, 6 μl 25 mmol MgCl_2 and 10 pmol of the sense primer, S1. Afterwards, denaturation at 95°C for 2 min was performed, followed by 30 cycles of amplification (each consisting of denaturation for 45 s at 94°C , annealing for 30 s at 64°C and extension for 1 min at 72°C). At the end, there was another extension for 7 min at 72°C . The 672 bp RT-PCR product was reamplified using the primers S2 and AS2. The reaction mix contained the RT-PCR product, 10 μl 10 $\times$ buffer, 8 μl 10 mol/l NTPs, 30 pmol S2 and 30 pmol AS2 primers, and 0.5 μl Taq polymerase (Perkin Elmer). The amplification product had an expected length of 296 bp.

Table 2 Primers used for RT-PCR and PCR (7).

Primer	Sequence
Sense primer S1 (nt 66 to 84)	5'-GCA GCA GCA GCG GCG GCA GCA G-3'
Sense primer S2 (nt 442 to 462)	5'-GAC AAA GTG ATG AGT AAA GTG-3'
Antisense primer AS2 (nt 719 to 738)	5'-TGT GGC CAT GCC AGC CAG CA-3'

nt = nucleotides.

The plasmid for the P450scc cDNA was provided by Dr WL Miller (University of California, San Francisco, CA, USA) (19).

Cell culture

The NCI-h295 tumour cell line (20), provided by AF Gazdar (National Cancer Institute, NIH, Bethesda, MD, USA), was maintained in TIS medium (RPMI 1640 medium supplemented with transferrin (0.1 mg/ml), insulin (5 mg/ml) and selenium (5.2 mg/ml)) as described by Fassnacht *et al.* (21). The cells were grown in 75 cm² flasks (approx. 1.0 million cells/ml), incubated for 48 h with forskolin (10⁻⁵ mol/l; Sigma, Deisenhofen, Germany), adrenocorticotrophin (ACTH) (10⁻⁸ mol/l; Ciba-Geigy, Wehr, Germany), aminoglutethimide (3 × 10⁻⁴ mol/l, Sigma) and metyrapone (3 × 10⁻⁴ mol/l, Sigma). Each experiment was performed in triplicate.

RNA extraction and Northern analysis

Polyadenylated RNA (tissue) and total RNA (cell culture) were isolated using commercial kits (Qiagen, Hilden, Germany). Three micrograms mRNA or 25 µg total RNA were electrophoresed on 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 membrane (Qiabran Nylon membrane, Qiagen) and crosslinked by exposure to UV radiation. Prehybridisation was carried out at 68 °C with Quickhyb solution (Stratagene, Heidelberg, Germany) for 1 h.

Hybridisation was performed in the same buffer at 68 °C for 1 h using the StAR cDNA labelled with [α^{32} -P]dCTP (Amersham Buchler, Braunschweig, Germany; Random Primed Labelling kit, Boehringer, Mannheim, Germany). Unincorporated nucleotides were separated from radiolabelled DNA probes by Nuc Trap purification columns (Stratagene). After washing at 60 °C twice for 15 min in 1 × SSC and for 15 min in 0.5 × 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. Blots were stripped with 0.1 × SSC and rehybridised with cDNA probes for P450scc, and for standardisation with a mouse β -actin cDNA probe.

The relative intensity of the hybridisation signals was quantified by scanning densitometry. Autoradiographic images were digitalised with a video camera and a Macintosh PowerMac 7100 computer-based image analysis system using the IMAGE program (NIH). For statistical analysis, the 1.6 kb StAR major transcript and the 2.0 kb P450scc transcript were selected. The results were expressed as a percentage of normal adrenal glands (=100%) or control cells (=100%) after normalisation for β -actin expression. Correlation was determined by linear regression analysis and expressed as Pearson's correlation coefficient.

Results

StAR mRNA was detected in all investigated adrenocortical tissues. Normal and neoplastic adrenocortical tissue expressed three StAR mRNA transcripts, one major band of 1.6 kb and two minor bands of 3.4 and 4.5 kb which were visible after prolonged exposure (data not shown).

There was only little variability in the magnitude of StAR mRNA expression in adrenocortical adenomas (APA: 80 ± 3%; CPA: 71 ± 10%; NEA: 64 ± 9.5% vs 100 ± 16% in normal adrenals; means ± S.E.M.), whereas adrenocortical carcinomas expressed low StAR mRNA levels (29 ± 9%) (Fig. 1A and B). P450scc mRNA expression, however, correlated with the endocrine activity of the adenomas, being higher in the aldosterone and cortisol producing adenomas than in non-functional adenomas (Fig. 1C). No close correlation was found between StAR mRNA and P450scc mRNA in adrenocortical tumours ($R^2 = 0.24$) (Fig. 1D).

Stimulation of the protein kinase A pathway with ACTH and forskolin induced a 1.2-fold and 1.5-fold increase, respectively, in the abundance of StAR mRNA expression in the NCI-h295 cell line (Fig. 2). The incubation of NCI-h295 tumour cells with the adreno-static compounds, aminoglutethimide and metyrapone, almost completely blocked steroidogenesis (aminoglutethimide: cortisol 1.3 ± 1%, dehydroepiandrosterone sulphate (DHEA-S) 8.2 ± 1%, aldosterone 6.1 ± 1%; metyrapone: cortisol 3 ± 1%, DHEA-S 11.5 ± 2%, aldosterone 9.9 ± 1% vs 100% in control cells). This resulted in a reduced expression of P450scc mRNA (control cells: 100 ± 3.9%; aminoglutethimide: 68 ± 0.9%; metyrapone: 69 ± 6.9%). There was no demonstrable effect on the expression of StAR mRNA (control cells: 100 ± 8%; aminoglutethimide: 94 ± 2%; metyrapone: 107 ± 11.5%).

Discussion

In this study, we investigated the expression and regulation of StAR mRNA in adrenocortical tumours and in the steroid producing NCI-h295 adrenocortical carcinoma cell line. Our data show that StAR mRNA expression is present in most of the adrenocortical tumour tissues analysed and shows little variability between tumours of different endocrine activity. StAR mRNA is also present in non-functional adenomas which express no or little P450scc mRNA.

Recent studies have implicated StAR as an essential component of the acute responses of steroidogenic cells to trophic hormones. StAR expression is restricted to steroidogenic cells, where it is rapidly induced by trophic hormone in a manner that correlates with the acute stimulation of steroidogenesis (5, 11, 22, 23). Definite proof for the essential role of StAR in steroidogenesis came from studies of patients with lipoid congenital adrenal hyperplasia, a congenital disorder characterised

Figure 1 (A) Northern blot of StAR mRNA and P450scc mRNA expression in human non-neoplastic adrenals and in adrenal tumours. The blots were stripped and rehybridised with a mouse β -actin cDNA (bottom). The panel shows the 1.6 kb StAR mRNA main transcript and the 2.0 kb P450scc mRNA transcript. (B) StAR mRNA expression assessed by Northern blot in normal adrenal glands (NAG), aldosterone producing adenomas (APA), cortisol producing adenomas (CPA), non-functional adenomas (NFA) and adrenocortical carcinomas (ACC). All values are expressed as a percentage of normal adrenal glands (=100%) and shown as means $\pm$ S.E.M. (C) Expression of P450scc mRNA expressed as a percentage of normal adrenal glands (=100%) and shown as means $\pm$ S.E.M. (D) Correlation between StAR mRNA and P450scc mRNA in adrenocortical tumours ($R^2 = 0.24$).

Figure 2 Northern blot of StAR mRNA and P450scc mRNA expression in NCI-h295 tumour cells. The figure shows the effects of incubation with ACTH or forskolin on StAR mRNA and P450scc mRNA expression. The blots were stripped and rehybridised with a mouse β -actin cDNA (bottom).

by severe impairment of steroid biosynthesis in the adrenal glands and gonads that is manifested both *in utero* and postnatally. Analysis of 3 patients with this disorder revealed mutations in the StAR gene that preclude the expression of functional StAR protein (6). In a more recent paper on 15 patients with lipoid congenital adrenal hyperplasia from 10 different countries, it was shown that the phenotype of this disease is the result of two separate events, an initial genetic loss of steroidogenesis that is dependent on StAR, and a subsequent loss of steroidogenesis that is independent of the protein due to cellular damage from accumulation of cholesterol ester (24).

We showed recently that adrenocortical tumours express P450 steroid enzymes dependent on the endocrine profile and the cellular differentiation of the tumours (25). For example, P450scc is upregulated in functional adrenal tumours, but is low in non-secreting adrenal tumours. Interestingly, the two main receptors of the adrenal cortex, the ACTH receptor and the angiotensin II type 1 receptor, follow a similar pattern of expression in adrenal tumours, with high levels of expression in functional adenomas, and low levels of gene expression in non-functional adenomas (26, 27). StAR mRNA expression in adrenocortical tumours was recently studied by Liu *et al.* (15). In their study they reported a good correlation between StAR and P450scc mRNA levels in normal and pathological adrenal tissues. This is in agreement with our data, with the exception of non-functional adenomas which did express StAR but not P450scc mRNA in our series. The difference between our results and the study of Liu *et al.* may be related to the fact that non-functional adenomas are heterogeneous as a group. Whereas some non-functional adenomas lack P450 enzymes and are truly non-functional, other tumours express P450 enzymes and may produce adrenal steroids in small amounts not sufficient to cause clinically apparent disease or to be detected by adrenal function tests. It is, therefore, our experience that P450scc mRNA correlates better with the endocrine

activity of the tumour tissue than does StAR mRNA. The difference in tumour-specific regulation of StAR and P450scc cannot be explained at present. It could be due to differences in the transcriptional regulation of both genes. So far, the promoter of the StAR gene has been only partially characterised (23, 28, 29). For basal gene transcription both promoters require binding sites of steroidogenic factor 1, and gene expression of both StAR and P450scc is increased by stimulation of the protein kinase A pathway. More recently, it was shown that the orphan nuclear receptor, DAX-1, binds to both promoters and suppresses gene transcription and steroidogenesis (30, 31).

In cell culture experiments of primary adrenocortical cells, Liu *et al.* showed homonymous upregulation of StAR and P450scc mRNA by ACTH and cAMP (15). We used the carcinoma cell line NCI-h295, which may serve as a pluripotent cellular model of the functional tumorous adrenal cortex. Our experiments demonstrate that stimulation of the protein kinase A pathway with ACTH or forskolin increased the gene expression of P450scc and StAR in a similar way. However, the fold stimulation induced by forskolin and by ACTH was weaker in the NCI cell line than that reported with primary adrenal cell cultures, probably due to low expression of ACTH receptor binding sites and an impaired adenylyl cyclase system. Our results are also at variance with the data of Rainey *et al.* (32) who showed that forskolin but not ACTH induced a strong increase in P450scc mRNA in the NCI-h295 cell line. These discrepancies could be due to differences in the experimental design (for example incubation for 24 h instead of 48 h, as in our experiments) or to differences in the NCI-h295 cell line used for the experiments. We have previously noted that the endocrine profile and the pattern of P450 enzyme expression of the NCI-h295 cell line changes during long-term culture conditions due to selection of different clones. With regard to P450scc expression, the NCI strain used in our experiments is known to respond well to stimulation by ACTH (21).

The incubation of the NCI-h295 tumour cells with the adrenostatic compounds, aminoglutethimide and metyrapone, resulted in suppression of glucocorticoid, mineralocorticoid and androgen secretion, paralleled by reduced expression of P450scc mRNA, suggesting presumably a positive feedback of steroid secretion on gene expression which is inhibited by adrenostatic treatment. Fassnacht *et al.* described a similar, but more profound effect on ACTH receptor mRNA expression (21). The expression of StAR mRNA was not changed by incubation with either aminoglutethimide or metyrapone in our experiments. This argues against a similar steroid-mediated feedback on StAR gene transcription.

In summary, we show that StAR mRNA expression is evenly expressed in benign functional and non-functional adrenocortical tumours. We conclude that

the functional state of these tumours cannot be explained by the relative abundance of StAR mRNA expression.

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