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The Acute and Chronic Effects of Adrenocorticotropin on the Levels of Messenger Ribonucleic Acid and Protein of Steroidogenic Enzymes in Rat Adrenal *in Vivo**

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ABSTRACT

The purpose of this study was to evaluate the effects of acute (a single injection) and chronic stimulation (twice daily injection for 9 days) by ACTH on changes occurring in the temporal expression of steroidogenic enzymes in the rat adrenal *in vivo*. Under acute ACTH stimulation, the level of steroidogenic acute regulatory protein (StAR) messenger RNA (mRNA) was increased within 0.5 h in both zona glomerulosa (ZG) and zona fasciculata-reticularis (ZFR), with maximal increases of 220–370% and 300–350% in the ZG and ZFR, respectively. Increases in the levels of StAR protein in homogenates were also found in the ZG (700%) and the ZFR (300%), but were delayed compared with those of their mRNA. Furthermore, the increase in mitochondrial StAR protein was concomitant with that in the homogenate, indicating that the entry of StAR into mitochondria might not be necessary to increase steroidogenesis during the early stimulatory phase. The levels of c-*jun*, c-*fos*, *jun*B, and *fos*B mRNA in ZG and ZFR were also rapidly maximally elevated within 0.5–1 h after ACTH administration and fell to near control levels 5 h posttreatment. The levels of c-*jun* protein were already increased in both zones at 1 h, reached 200% at 3 h, and remained elevated 5 h post-ACTH treatment. The levels of c-Fos protein were maximally increased by 240% in both zones after 1 h and decreased thereafter to control values at 5 h. Few changes were observed in the adrenal protein contents of cholesterol side-chain cleavage cytochrome P450 (P450scc), cytochrome P450 11β-hydroxylase (P450C11), cytochrome P450 21-hydroxylase (P450C21), and 3β-hydroxysteroid dehydrogenase (3βHSD). Under chronic stimulation by ACTH, we observed elevations in the levels of plasma corticosteroids and changes in the mRNA and protein levels of many adrenal steroidogenic enzymes in both zones. In the ZG, administration of ACTH for 9 days provoked an increase in the level of StAR mRNA (210–270%) and a decrease in the levels of 3βHSD, cytochrome P450 aldosterone synthase (P450aldo), and AT_1 receptor mRNA (by 40%, 70%, and 90%, respectively), whereas the levels of P450scc and P450C21 mRNA did not differ significantly from the control values. Western blotting analysis showed that the adrenal ZG protein levels of StAR and P450scc were increased (150%), 3βHSD was not changed, and P450C21 was decreased by 70%. In the ZFR, the levels of P450scc and StAR mRNAs were increased (260% and 570–870%, respectively). The levels of 3βHSD, P450C21, and P450C11 mRNA did not differ from control values in that zone. Western blotting analysis showed that the ZFR protein level of 3βHSD was not changed, P450scc and P450C21 were decreased by 40% and 60%, respectively, and StAR was increased by 160%. Although c-*fos* and *fos*B mRNAs were undetectable after 9 days of chronic ACTH treatment, c-*jun* mRNA and its protein were still detectable, suggesting a basic role for this protooncogene in maintaining the integrity and function of the adrenal cortex. When dexamethasone was administered to rats for 5 days to inhibit their ACTH secretion, the mRNA levels of many steroidogenic enzymes were decreased, with the exception of StAR, 3βHSD, and P450aldo. These results confirm the importance of physiological concentrations of ACTH in maintaining normal levels of adrenocortical enzymes and also indicate that in addition to ACTH, other factors are involved in controlling the expression of StAR, 3βHSD, and P450aldo.

In conclusion, we showed that ACTH acutely increases StAR mRNA followed, after a delay, by an increase in the level of StAR protein; this suggests that posttranslational modifications of the StAR precursor occurred during the early stimulatory phase and before the apparent translation of the newly formed mRNA. The rapid induction of protooncogenes suggests their participation in the action of ACTH to stimulate steroidogenesis. Under chronic stimulation by ACTH, adrenals were hypertrophied, and the expression of many steroidogenic enzymes was modified, particularly the level of StAR protein was increased in the ZG and ZFR, confirming the importance of this protein in the control of steroidogenesis in a situation similar to that of Cushing's syndrome. (*Endocrinology* **139:** 3913–3922, 1998)

THE BIOSYNTHESIS of corticosteroids involves the participation of various steroidogenic enzymes (1). Cholesterol side-chain cleavage cytochrome P450 (P450scc), associated with the inner mitochondrial membrane, catalyzes the transformation of cholesterol to pregnenolone. This reaction was long believed to be the rate-limiting step in steroidogenesis (2, 3). It was recently reported that this limiting step was not due to P450scc activity but, rather, to the mobilization of cholesterol and its transfer across the mitochondrial membrane (4). Indeed, the transfer of cholesterol into mitochondria across the intermembrane aqueous space was shown to be inhibited by cycloheximide, an inhibitor of protein synthesis that also concomitantly blocked steroidogenesis (5, 6). Clark *et al.* (4) recently reported that expression of the steroidogenic acute regulatory (StAR) protein induced steroidogenesis without hormonal stimulation. Furthermore, in lipoid congenital adrenal hyperplasia, which is characterized by lipoidal accumulation and impaired gonadal and adrenal steroidogenesis, mutations in the StAR gene were shown to be responsible for defective steroidogenesis (7–12). This biochemical and genetic evidence has indicted StAR protein to be a key molecule in controlling cholesterol transfer across the mitochondrial membrane and conse-

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quently in controlling the transformation of cholesterol to pregnenolone.

Among steroidogenic enzymes, 3β-hydroxysteroid dehydrogenase (3βHSD) is responsible for the transformation of Δ^5 to Δ^4 steroids, and cytochrome P450 21-hydroxylase (P450C21) is responsible for the catalysis of progesterone to yield deoxycorticosterone (1). In most mammalian species studied to date, deoxycorticosterone has been shown to be transformed into aldosterone in the adrenal zona glomerulosa (ZG) by cytochrome P450 aldosterone synthase (P450aldo), which possesses both 11β-hydroxylase and 18-methyloxidase activities (1). In the zonae fasciculata and reticularis (ZFR), cytochrome P450 11β-hydroxylase (P450C11) catalyzes the transformation of deoxycorticosterone to corticosterone and that of 11-deoxycortisol to cortisol.

The regulation of steroidogenesis by ACTH is mediated by cAMP, which produces acute effects occurring within minutes and chronic effects that require hours before being discernible (1). In a preliminary report (13) we have also shown that administration of ACTH to rats produced, within a few minutes, increases in the messenger RNA (mRNA) levels of adrenal c-*jun*, c-*fos*, *jun*B, and *fos*B, suggesting that these protooncogenes might play a role in the acute stimulation by ACTH.

The purpose of this study was to examine the short-term (acute) and long-term (chronic) effects of ACTH administration on temporal changes occurring in the expression of rat adrenal P450s, 3βHSD, StAR protein, and protooncogenes of the Jun and Fos family. Dexamethasone was also administered to determine the effect of the inhibition of ACTH secretion on the expression of the above-mentioned adrenal components.

We found that acute stimulation by ACTH rapidly changed the expression of the genes of the *jun/fos* family in the ZG and the ZFR. Under such conditions, the expression of steroidogenic enzyme proteins was little affected, with the exception of StAR; the level of StAR mRNA was increased, followed by a delayed increase in the level of its protein. Our results indicate that during the first hour after ACTH stimulation, StAR protein does not accumulate in the mitochondrion and, therefore, could act from the external surface of the organelle. Under chronic stimulation by ACTH, the levels of StAR protein in both ZG and ZFR were elevated, showing the importance of this protein in the control of steroidogenesis in a situation that mimics Cushing's syndrome. Dexamethasone treatment perturbed the expression of many steroidogenic enzymes, thus confirming the importance of ACTH in maintaining the functional status of the adrenal cortex.

Materials and Methods

Materials

^{32}P-Labeled radiochemicals were purchased from Amersham Canada (Oakville, Canada). Acthar and Acthar Gel were obtained from Armour Pharmaceutical Co. (Phoenix, AZ), and Synacthen Depot was obtained from Ciba Pharmaceuticals, Division of Ciba-Geigy Canada (Mississauga, Canada).

Animals and treatments

Two-month-old male Long-Evans rats were purchased from Charles River (St. Constant, Canada). Rats received a single treatment of ACTH consisting of 4 U Acthar (for rapid action) and 2 U Acthar Gel/250 g BW, or two daily injections of Synacthen Depot (7.5 U/250 g BW) for a sustained action. Dexamethasone acetate (400 μg/250 g BW) was injected once daily. Controls received vehicle only. Animals were killed by decapitation (in accordance with the ethical standards of the institutional review committee) at different times after the first injection, as specified in *Results*. Blood was collected, and adrenals were removed. The zona glomerulosa was separated from the zonae fasciculata and reticularis containing the medulla by the method of Giroud *et al.* (14).

RNA extraction from tissues and Northern blotting analysis

Total RNA from rat adrenal ZG and ZFR was extracted using the Tri-Reagent protocol (Molecular Research Center, Cincinnati, OH). RNA (15 μg) samples were denatured with glyoxal (15), and then fractionated by electrophoresis on a diethylpyrocarbonate-treated 1% agarose gel in 0.01 M phosphate buffer, pH 7.0. The fractionated RNA was transferred to positively charged nylon membranes (Boehringer Mannheim, Mannheim, Germany), which were then hybridized for 16 h at 42 C with the following α-^{32}P-labeled probes: 1) bovine P450scc and P450C21 complementary DNAs (cDNAs) obtained from Dr. M. R. Waterman (Vanderbilt University, Nashville, TN); 2) rat 3βHSD cDNA (16); 3) hamster StAR cDNA (17); 4) hamster P450C11 cDNA (18); 5) oligonucleotide sequence specific to the rat P450aldo (19); 6) v-*fos* from Oncor (Gaithersburg, MD); 7) c-*jun*, *jun*B, and *fos*B cDNAs obtained from the American Type Culture Collection (Rockville, MD); 8) and bovine adrenal angiotensin II receptor type 1 (AT_1) cDNA (20). All blots were also analyzed with an 18S cDNA probe (American Type Culture Collection), and the results obtained were used to standardize quantities of mRNA. Autoradiograms were observed by exposing the blots to Kodak X-Omat RP films (Eastman Kodak, Rochester, NY) with Cronex Lightning plus enhancing screens (DuPont Cronex, Wilmington, DE). The intensity of bands on the films was determined using an LKB 2222–020 Ultroscan XL laser densitometer (Pharmacia Canada, Baie d'Urfe, Canada). In some experiments radioactivity was detected using an optical imager (PhosphorImager SF, Molecular Dynamics, Sunnyvale, CA).

Immunoblotting

Homogenates of rat adrenal ZG and ZFR (15) were analyzed by immunoblotting as previously described (21). Tissues were homogenized in 50 mM Tris (pH 7.4), 0.25 M sucrose, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 0.1 mM leupeptin, 30 mM iodoacetamide, and 0.125 μM aprotinin using a Potter-Elvehjem homogenizer with a loosely fitting Teflon pestle. A portion of the homogenate was centrifuged for 10 min at 900 × *g*, and the supernatant was then centrifuged for 15 min at 9500 × *g*. Mitochondrial pellet was resuspended in homogenization buffer. Cholate and SDS were added to homogenate and mitochondrial fractions to final concentrations of 1% and 0.1%, respectively. Preparations were frozen in liquid nitrogen, thawed twice, and solubilized in Laemmli sample buffer (22). They were passed through a 26-gauge needle, then boiled for 5 min, and finally centrifuged at 12,000 × *g* for 2 min. Soluble proteins, 50 μg of homogenate and mitochondrial preparations, were electrophoresed on 10% polyacrylamide gel in the presence of 0.1% SDS (SDS-PAGE) and analyzed by immunoblotting as previously described (21, 23) using a rabbit polyclonal antimouse StAR peptide antibody provided by Dr. D. M. Stocco (Department of Cell Biology and Biochemistry, Texas Tech University, Health Sciences Center, Lubbock, TX). Antibodies used to analyze c-*jun* and c-*fos* were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other antibodies against other steroidogenic enzymes were the same as those used in a previous work (23). Immunoreactive proteins were detected using ECL light-emitting reagents (Amersham International, Aylesbury, UK). Autoradiograms were observed by exposing the blots to Kodak X-Omat RP films. The intensity of bands on the films was determined using the above-mentioned laser densitometer.

Steroid analysis

Corticosterone and aldosterone analyses were performed as previously described (15).

Statistical analysis

Differences between mean mRNA or protein levels were analyzed by ANOVA followed by Dunnett's test, using the SigmaStat program for Windows (Jandel Corp., San Rafael, CA).

Results

Short-term effect of ACTH on plasma corticosterone and aldosterone

When rats were given short acting ACTH, their plasma corticosterone and aldosterone levels were maximally elevated between 0.5–1 h to decrease thereafter, but they were still more elevated than the control levels 5 h postinjection (Table 1).

Short-term effect of ACTH on StAR mRNA and on StAR protein

As shown in Fig. 1, ACTH administration to rats provoked increases in the levels of StAR mRNA in adrenal ZG and ZFR; changes in the level of StAR mRNA bands were observed as early as 30 min after treatment; levels were maximally elevated between 1 and 3 h, with increases of 220% (for the 1.6-kb band) and 370% (for the 3.5-kb band) in the ZG and of 300% (for the 1.6-kb band) and 350% (for the 3.5-kb band) in the ZFR. Although ACTH administration always induced a rapid increase in the adrenal level of StAR mRNA, the order of magnitude varied from one experiment to another (data not shown). Dexamethasone had no effect on the adrenal StAR mRNA level 3 h after its administration. Furthermore, the coadministration of dexamethasone with ACTH did not block the increases induced by ACTH (results not shown).

Western blotting analysis revealed that the adrenal content of StAR protein was increased in both ZG and ZFR after ACTH administration. However, in contrast to the rapid increase in the level of StAR mRNA (within 30 min; see Fig. 1) after ACTH administration, there was a delay before an increase in the StAR protein level occurred. This is well illustrated in Fig. 2 and Table 2a, which show that the level of StAR protein was unchanged at 1 h and was increased only 3–5 h after ACTH treatment in homogenates of both ZG and ZFR preparations. We also determined whether StAR protein accumulated more rapidly in mitochondria than in homogenates. Figure 2 and Table 2a show that mitochondrial preparations of both zones were enriched in StAR protein at the same time as for homogenate preparations, but not before.

TABLE 1. Effects of ACTH and dexamethasone (DEX) administration on plasma corticosterone (Cpd B) and aldosterone (Aldo)

Treatment	Cpd B (μg/dl)	P^a	Aldo (ng/dl)	P^a
Control	1.2 ± 0.4		13.6 ± 4.4	
ACTH, 30 min	49.8 ± 5.8	*	68.2 ± 1.3	*
ACTH, 1 h	53.2 ± 7.6	*	78.5 ± 8.3	*
ACTH, 3 h	38.0 ± 8.6	*	61.7 ± 7.9	*
ACTH, 5 h	31.5 ± 7.3	*	40.4 ± 4.4	*
DEX, 3 h	0.2 ± 0.1	NS	4.3 ± 0.3	NS
Control	1.2 ± 0.4		13.6 ± 4.4	
ACTH, 12 h	61.2 ± 8.2	*	84.9 ± 8.2	*
ACTH, 24 h	67.2 ± 20.5	*	97.6 ± 1.6	*
ACTH, 36 h	53.2 ± 16.8	*	71.5 ± 7.4	*
ACTH, 36 + 24 h	2.4 ± 0.7	*	2.1 ± 0.7	NS
Control	1.6 ± 0.9		9.3 ± 1.5	
ACTH, 9 days	53.2 ± 8.7	*	46.2 ± 1.0	*
DEX, 5 days	0.24 ± 0.1	NS	6.7 ± 1.2	NS

Data are expressed as the mean ± SEM (n = 3 determinations).
[a] Comparison between experimentals and controls: *, $P < 0.05$.

Short-term effect of ACTH on the levels of P450s and 3βHSD proteins

We studied the short-term effect of ACTH on the adrenal contents of P450scc, P450C21, P450C11, and 3βHSD proteins as analyzed by Western blotting. No or only small changes were observed in the levels of the four proteins in the ZG and ZFR after ACTH administration (Fig. 3 and Table 2).

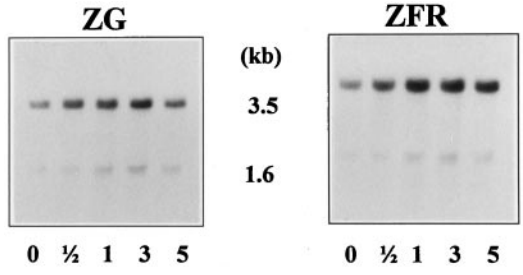

FIG. 1. A time study of the effects of ACTH on the level of StAR mRNA in rat adrenal zona gomerulosa (ZG) and zonae fasciculata and reticularis (ZFR) containing the medulla. Five groups of male rats received an intramuscular injection of a fast acting ACTH preparation and these animals were killed at 0, 1/2, 1, 3, and 5 h after treatment.

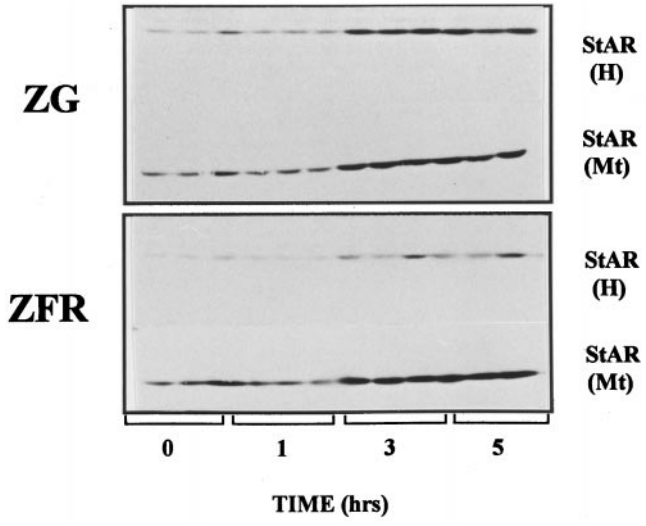

FIG. 2. A time study of the effects of ACTH on the level of StAR protein in rat adrenal. Western blotting analysis were performed on zona glomerulosa (ZG) and zonae fasciculata and reticularis (ZFR) containing the medulla. Groups of male rats were injected with the fast acting ACTH preparation and they were then killed at 0, 1, 3, and 5 h after treatment. Analyses were performed on 50 μg of homogenate (H) or mitochondrial (Mt) proteins.

TABLE 2a. Short-term effects of ACTH treatment on StAR and P450scc proteins as analyzed by Western blotting

Time (h)	Homogenate	P^a	Mitochondria	P^a
StAR protein				
ZG				
0	1.00 ± 0.40		1.00 ± 0.34	
1	1.01 ± 0.13	NS	0.62 ± 0.03	NS
3	7.01 ± 0.66	*	3.49 ± 0.38	*
5	5.95 ± 0.94	*	4.04 ± 0.45	*
ZFR				
0	1.00 ± 0.32		1.00 ± 0.28	
1	1.25 ± 0.39	NS	0.45 ± 0.05	NS
3	3.03 ± 0.56	NS	1.57 ± 0.13	NS
5	4.13 ± 1.06	*	2.09 ± 0.30	*
P450scc				
ZG				
0	1.00 ± 0.02		1.00 ± 0.05	
1	1.07 ± 0.01	NS	0.95 ± 0.04	NS
3	1.17 ± 0.001	*	0.93 ± 0.11	NS
5	0.99 ± 0.05	NS	0.91 ± 0.02	NS
ZFR				
0	1.00 ± 0.03		1.00 ± 0.01	
1	1.01 ± 0.02	NS	1.02 ± 0.02	NS
3	0.97 ± 0.03	NS	1.01 ± 0.02	NS
5	0.88 ± 0.02	*	0.97 ± 0.02	NS

Fifty micrograms of proteins were used per sample. Control values were arbitrarily fixed at 1.00. Data were expressed as the mean ± SEM (n = 3).

[a] Comparison between experimentals and controls: *, $P < 0.05$.

TABLE 2b. Short-term effects of ACTH treatment on P450C11, P450C21, 3β-HSD, c-*jun*, and c-*fos* as analyzed by Western blotting

Time (h)	Homogenate	P^a	Homogenate	P^a
P450C11				
ZG			ZFR	
0	1.00 ± 0.004		1.00 ± 0.08	
1	0.98 ± 0.02	NS	1.07 ± 0.02	NS
3	1.01 ± 0.02	NS	1.16 ± 0.03	NS
5	0.87 ± 0.01	*	1.09 ± 0.03	NS
P450C21				
ZG			ZFR	
0	1.00 ± 0.10		1.00 ± 0.09	
1	0.97 ± 0.03	NS	0.96 ± 0.07	NS
3	1.06 ± 0.01	NS	1.09 ± 0.09	NS
5	1.07 ± 0.03	NS	1.24 ± 0.13	NS
3βHSD				
ZG			ZFR	
0	1.00 ± 0.04		1.00 ± 0.02	
1	1.05 ± 0.01	*	1.05 ± 0.03	NS
3	1.05 ± 0.01	*	1.10 ± 0.03	NS
5	1.03 ± 0.001	NS	1.14 ± 0.01	*
c-*jun*				
ZG			ZFR	
0	1.00 ± 0.05		1.00 ± 0.07	
1	1.35 ± 0.10	NS	1.29 ± 0.05	*
3	1.99 ± 0.08	*	2.01 ± 0.06	*
5	1.97 ± 0.16	*	2.53 ± 0.36	*
c-*fos*				
ZG			ZFR	
0	1.00 ± 0.09		1.00 ± 0.04	
1	2.42 ± 0.11	*	2.38 ± 0.27	*
3	1.66 ± 0.19	*	1.55 ± 0.10	NS
5	1.41 ± 0.10	NS	1.69 ± 0.20	NS

Fifty micrograms of proteins per sample. Control values were arbitrarily fixed at 1.00. Data were expressed as the mean ± SEM (n = 3).

[a] Comparison between experimentals and controls: *, $P < 0.05$.

Short-term effect of ACTH on protooncogenes of the jun/fos family

Figure 4 shows results confirming our preliminary findings from a first series of experiments (13) that the mRNA levels of c-*jun*, c-*fos*, *jun*B, and *fos*B were all increased within 30 min after ACTH administration in both adrenal ZG and ZFR. These high levels were not sustained, as they were already diminished after 3 h and returned to near or below basal values at 5 h. Similar results were found in a third series of experiments (results not shown). The coadministration of dexamethasone with ACTH did not prevent the action of the hormone to increase the levels of the protooncogenes. Western blotting analysis revealed that the ZG and ZFR contents in c-*jun* and c-*fos* were increased 1 h after ACTH administration (Table 2b and Fig. 3).

Long-term effect of ACTH on plasma corticosterone and aldosterone

With twice daily administration of long acting ACTH, the levels of plasma corticosterone and aldosterone remained maximally elevated at 12 h, 24 h, 36 h, and 9 days after the first injection (Table 1). When ACTH was administered for 36 h, and animals were killed 24 h later, the levels of plasma corticosterone and aldosterone in these latter groups were decreased to near control values.

Long-term effect of ACTH on StAR mRNA and protein

Compared with controls, the twice daily administration of the long acting ACTH preparation resulted in levels of StAR mRNA that were elevated at 12 h in the ZG (310% for the 1.6-kb band, and 290% for the 3.5-kb band) and in the ZFR (410% for the 1.6-kb band, and 490% for the 3.5-kb band); these levels remained elevated at 24 and 36 h after the first injection (Fig. 5). When rats were injected for 36 h and killed 24 h after the last injection, the levels of StAR mRNA had returned to or near basal values. In another series of experiments (Fig. 6 and Table 3), we found that after 9 days of ACTH treatment, StAR mRNA levels remained elevated in the ZG (210% for the 1.6-kb band and 270% for the 3.5-kb band) and the ZFR (870% for the 1.6-kb band and 570% for the 3.5-kb band). After 5 days of dexamethasone treatment, the StAR mRNA levels did not change significantly in the ZG (Table 3) and were elevated in the ZFR (450% for the 1.6-kb band and 300% for the 3.5-kb band).

Western blotting analysis revealed that the adrenal content of StAR protein was increased in both ZG (150%) and ZFR (160%) after ACTH administration (Fig. 7 and Table 4). No significant changes occurred in adrenal StAR protein content under dexamethasone treatment for 5 days.

Long-term effect of ACTH on expression of the protooncogenes Jun and Fos

Figure 6 and Table 3 show that ACTH administration to rats for 9 days resulted in a decrease in the c-*jun* mRNA level in the ZG. A faint *jun*B mRNA band was detected in the ZG and the ZFR, this did not appear to vary during chronic ACTH treatment (results not shown). c-*fos* and *fos*B mRNAs were not detected. During dexamethasone treatment for 5 days, *jun*B mRNA was significantly increased by 400% in the

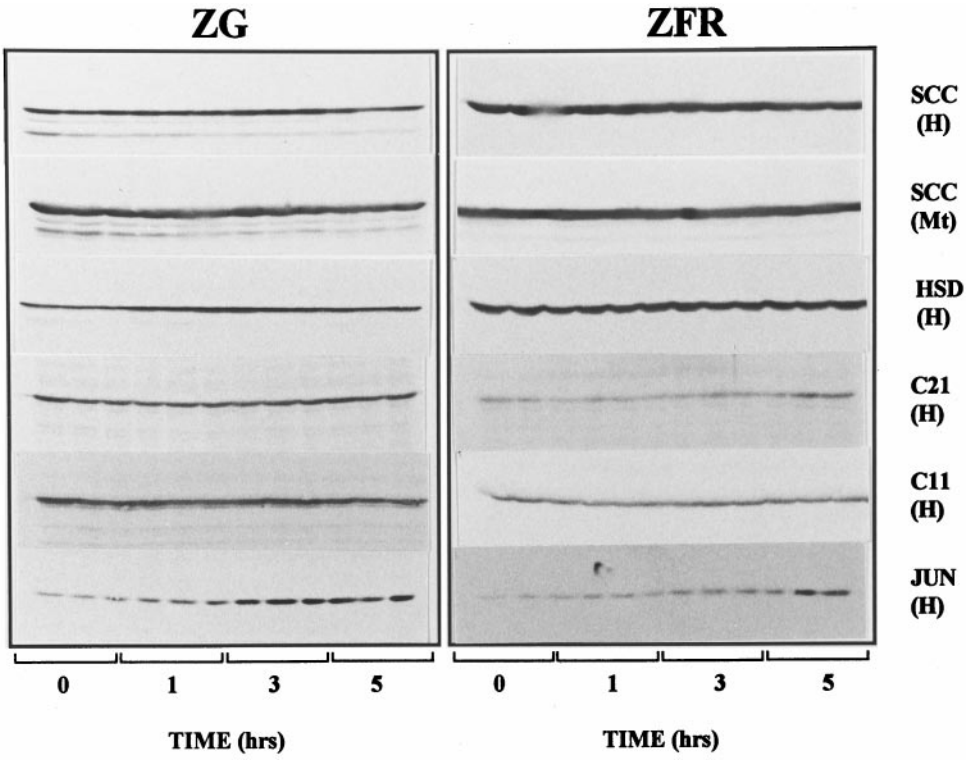

FIG. 3. Western blotting analyses on rat adrenal zona glomerulosa (ZG) and zonae fasciculata and reticularis (ZFR) containing the medulla. Groups of male rats were injected with the fast acting ACTH preparation, and they were then killed at 0, 1, 3, and 5 h after treatment. Analyses were performed on 50 μg of homogenate (H) or mitochondrial (Mt) proteins. SCC, P450scc; HSD, 3β-HSD; C21, P450C21; C11, P450C11; Jun, c-*jun*.

ZFR (results not shown). The level of c-*jun* mRNA did not change during dexamethasone treatment in the ZG and ZFR. Western analysis shows that the level of c-Jun protein was increased by 190% in the ZG and remained unchanged in the ZFR (Fig. 7 and Table 4).

Long-term effect of ACTH on steroidogenic enzyme mRNAs

Nine days of ACTH administration provoked changes in the levels of some adrenal steroidogenic enzyme mRNAs in both ZG and ZFR (Fig. 6 and Table 3). In the ZG, a decrease in the mRNA levels of 3βHSD was observed, with no significant changes in those of P450scc and P450C21. The level of AT_1 receptor mRNA was decreased by 90% in the ZG, similarly to that of P450aldo mRNA (70%). In the ZFR, the mRNA of P450scc was significantly increased (260%), whereas those of P450C11, P450C21, and 3βHSD were not significantly changed.

When endogenous ACTH secretion was inhibited by daily injections of dexamethasone for 5 days, and the rats were killed 24 h after the last injection, the level of P450scc mRNA in the ZG was decreased by 76%, whereas the levels of P450C21, P450aldo, and 3βHSD were similar to control values (Table 3 and Fig. 6). The level of AT_1 receptor mRNA was not significantly changed in the ZG by dexamethasone treatment. In the ZFR, the levels of P450scc, P450C21, and P450C11 mRNA were decreased by 90%, 60%, and 98%, respectively, whereas that of 3βHSD mRNA was slightly increased (120%). These results clearly show that inhibition of ACTH secretion affected the levels of many steroidogenic enzyme mRNAs in both ZG and ZFR, and therefore, they suggest an important role for ACTH in maintaining these adrenal zones in a functional state.

Western blotting analysis revealed that, when expressed on a milligram of protein basis, the content of P450scc protein was increased in the ZG and was decreased in the ZFR after 9 days of ACTH treatment (Fig. 7, and Table 4). The content of P450C11 protein was not significantly changed in the ZFR after 9 days of ACTH treatment (data not shown). The content of P450C21 protein was decreased in the ZG and the ZFR. No significant changes occurred in the 3βHSD protein of either zones.

Dexamethasone treatment for 5 days resulted in significant decreases in the level of P450C21 in the ZG and no changes in the ZFR. The levels of P450scc and 3βHSD were not changed in either zone by dexamethasone treatment.

Discussion

In this work we studied the short and long-term effects of ACTH administration on plasma corticosteroids and adrenal components related to the steroidogenesis in rats. Over the short-term, ACTH induced rapid changes in the levels of plasma corticosterone and aldosterone, adrenal StAR protein, and adrenal protooncogenes of the *jun/fos* family, but had little effect on adrenal P450s and 3βHSD enzymes. In contrast, long-term ACTH treatment of rats provoked profound changes in the levels of plasma corticosteroids and many steroidogenic enzymes in adrenal ZG and ZFR.

Acute ACTH stimulation provoked a rapid increase in the levels of plasma corticosteroids and StAR mRNA within 0.5–1 h. The newly transcribed StAR mRNA was apparently not immediately translated into protein, as the ZG and ZFR StAR protein contents did not differ between preparations from 1-h treated and control animals. There was thus a delay between the increase in the levels of StAR mRNA and StAR

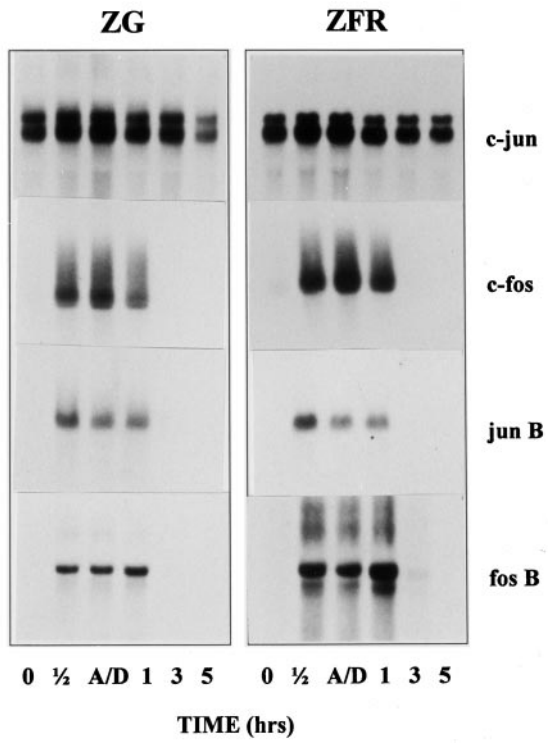

FIG. 4. A time study of the effects of ACTH on the level of proto-oncogenes of the Jun/Fos family. Northern blotting analyses were performed on rat adrenal zona glomerulosa (ZG) and zonae fasciculata and reticularis (ZFR) containing the medulla. Groups of male rats were injected with the fast acting ACTH and animals were then killed at 0, 1/2, 1, 3, and 5 h after treatment. Another group received ACTH + dexamethasone (A/D) and rats were killed Ωh after treatment. Analyses were performed on 15 μg of total RNA using c-*jun*, c-*fos*, jun B, and fos B cDNAs as probes.

protein. Clark *et al.* (24), using an *in vitro* model, found that progesterone production, StAR mRNA, and StAR protein expression were concomitant in MA-10 cells stimulated by $(Bu)_2$cAMP after a lag period of 30 min. Clark *et al.* (24) also found a spatial and temporal relationship between StAR protein expression and the capacity to produce steroid hormones *in vivo*, although they did not determine whether StAR protein synthesis occurred concomitantly or was slightly retarded compared with the expression of StAR mRNA. In our *in vivo* study, the delay observed between the increase in the level of StAR mRNA and that in its protein might be tentatively explained by the fact that the rat adrenal cortex cells, under acute stimulation, might be using existing StAR protein precursor for their immediate function before apparently translating newly transcribed StAR mRNA; this situation might be different in unstimulated MA-10 cells because they have an extremely low basal level of StAR protein (25). Supporting our findings, in response to tropic hormones, precursor StAR protein undergoes several post-translational modifications to yield mature 30-kDa proteins (6, 26–30). Moreover, previous studies showed that actinomycin D did not inhibit hormonal stimulation of corticosterone production when administered to rats or added *in vitro* to rat adrenal quarters or slices (31–33), suggesting that transcription may not be required for the hormonally induced acute steroidogenic response.

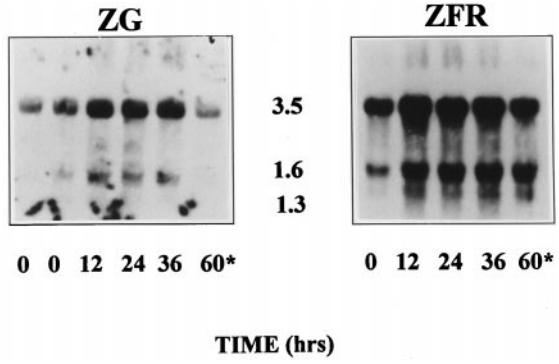

FIG. 5. A time study of the long-term effects of ACTH on the levels of StAR mRNA in rat adrenal zona gomerulosa (ZG) and zonae fasciculata and reticularis (ZFR) containing the medulla. Five groups of rats were injected twice daily with the long acting ACTH preparation and they were killed at 0, 0, 12, 24, and 36 h after the first treatment; another group received three injections of ACTH and rats were killed 60 h (60*) after the first treatment.

Surprisingly, the StAR protein content of mitochondria increased at the same time as that of homogenates. This indicates that during the first hour after ACTH administration, although steroidogenesis was maximally stimulated, StAR protein did not accumulate in the mitochondria. Hence, it can be hypothesized that it is not necessary for StAR protein to enter the mitochondria to increase the transport of cholesterol leading to increased steroidogenesis. This hypothesis is in agreement with results reported by Arakane *et al.* (34), which found that deletion of up to 62 amino acid residues of StAR protein from the N-terminus did not affect its steroidogenic-enhancing activity, but prevented its importation into mitochondria. Arakane's results and the fact that StAR protein was not accumulated into mitochondria when steroidogenesis was maximally stimulated imply that StAR protein acts on the external surface of the mitochondria during the acute enhanced steroidogenesis phase.

Although ACTH had a short-term enhancing effect on the level of StAR protein, only small changes occurred in the P450scc protein level in the ZFR within 3 h, with a small decrease at 5 h and a small increase at 3 h for the ZG. A similar situation was reported in the hamster, where ACTH administration did not affect the adrenal P450scc protein content (23). In rat ovaries treated *in vivo* with hCG, Sandhoff and McLean (35) reported that in contrast to the dramatic increase in the expression of StAR mRNA, P450scc mRNA levels remained unchanged in response to hormonal stimulation; this agrees with our findings. Also in agreement with results reported for hamster adrenal, acute ACTH stimulation did not greatly affect the levels of P450C21, P450C11, and 3βHSD proteins in the rat adrenal. Taken together, these results indicate that corticosteroidogenesis is not controlled by changes in the levels of adrenal P450 and 3βHSD enzymes under acute ACTH stimulation.

As recently reviewed (1), there is good evidence that the members of the *jun/fos* oncogene family play a role in the mechanism of action of ACTH in the adrenal. Indeed, a circadian variation in the expression of c-*fos* was found in rat adrenal ZFR using immunocytochemistry (36). Also the level of c-*fos* was rapidly increased after ACTH administration in

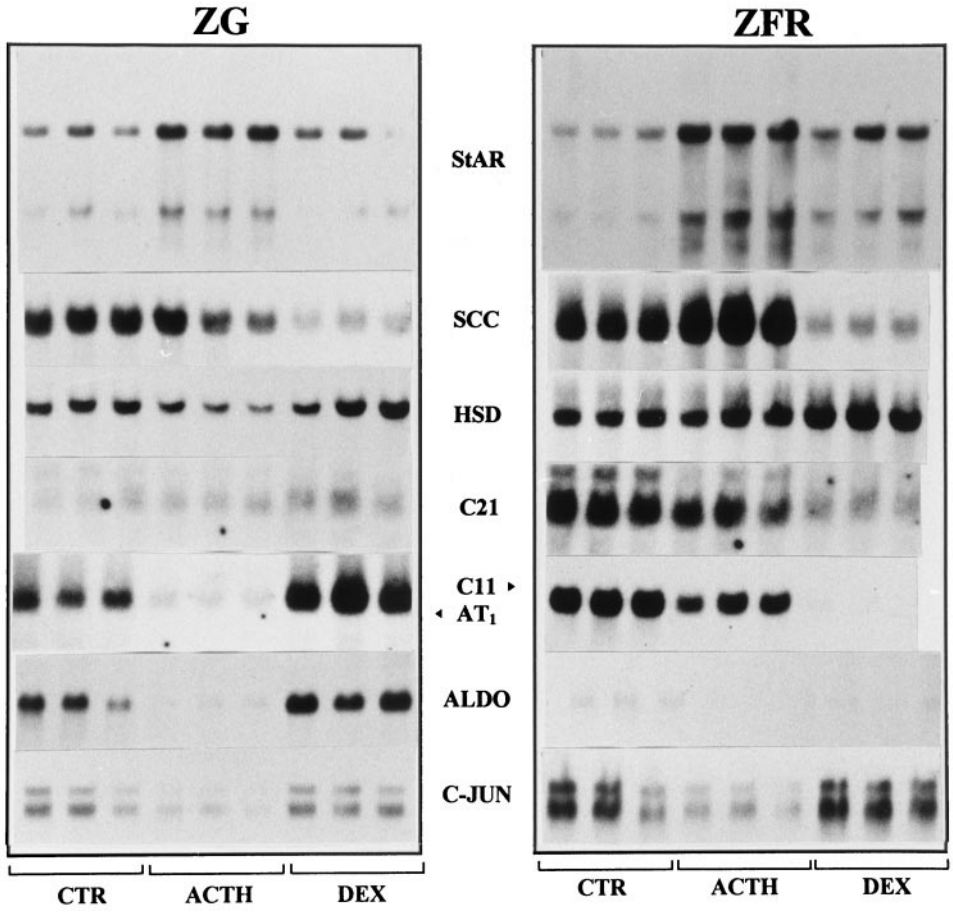

FIG. 6. Long-term effects of ACTH on rat adrenal mRNAs. Northern blotting analyses were performed on zona glomerulosa (ZG) and zonae fasciculata and reticularis (ZFR) containing the medulla. Three groups of male rats were injected twice daily with the long acting ACTH preparation for 9 days, and they were killed 12 h after the last treatment; three additional groups received a daily injection of dexamethasone acetate (Dex) for 5 days, and they were killed 24 h after the last treatment. Three controls groups were daily injected with 0.15 M NaCl (CTR). Analyses were performed on 15 μg of total RNA. StAR, StAR protein; SCC, P450scc; HSD, 3β-HSD; C21, P450C21; C11, P450C11; AT_1, Angiotensin II receptor type 1; ALDO, P450aldo; C-JUN, c-*jun*.

the adrenal cortex of intact (37) and hypophysectomized rats (38). Viard *et al.* (39) also reported rapid increases in the levels of *jun*B, c-*fos* and c-*jun* mRNA after the addition of ACTH to incubation medium of bovine and ovine adrenal cells in culture. In agreement with the studies mentioned above, in this work we found that administration of ACTH to rats induced a rapid increase in the level of c-*fos* mRNA and in other early response genes, c-*jun*, *jun*B, and *fos*B; furthermore, these mRNAs were translated into proteins, as increases in adrenal ZG and ZFR contents in c-*jun* and c-*fos* proteins were observed as early as 1 h after ACTH administration. As previously mentioned, the level of StAR mRNA began to increase in the adrenal ZG and ZFR as early as 30 min after ACTH treatment and continued to increase up to 3–5 h, whereas the mRNA levels of protooncogenes c-*jun*, c-*fos*, and *fos*B were already maximally increased at 0.5–1 h and those for *fos*B were maximally increased at 1–3 h, then decreased thereafter to near control values by 3–5 h. Taken together, these results suggest that under physiological conditions, early response genes of the *jun/fos* family may participate in the induction of StAR expression.

Chronic stimulation by ACTH changed the level of StAR protein, but in contrast to the acute stimulation, the long-term treatment also changed the levels of other steroidogenic enzymes in rat adrenals. Under such conditions, the levels of StAR mRNA were significantly elevated in the ZG (210–270%) and ZFR (450–870%), and the levels of StAR protein (expressed on a milligram of protein basis) were also significantly elevated in the ZG (150%) and ZFR (160%), indicating the importance of StAR protein in maintaining a high level of steroidogenesis in a situation similar to that which prevails in Cushing's syndrome. In agreement with our results, ACTH administration to hypophysectomized rats resulted in an increase in adrenal StAR mRNA 24 h after treatment (40). In H295R adrenocortical cells, $(Bu)_2$cAMP induced StAR protein expression (41).

There was not always a correlation between the levels of mRNA and protein of steroidogenic enzymes. In fact, there was no correlation between the levels of mRNA and protein for P450scc in adrenals of rats who had received ACTH for 9 days. Indeed, in the ZG, the level of P450scc mRNA was not changed, whereas its protein level was significantly increased by 150%; in the ZFR, the level of P450scc mRNA was increased to 260%, whereas its protein level was decreased to 56%. P450scc mRNA was significantly increased in whole adrenals of rats treated with ACTH for 3 days (42). Sander *et al.* (43) found no changes in the level of P450scc mRNA in adrenals of Sprague-Dawley rats after 8 days of ACTH treatment. *In vitro*, ACTH stimulation of adrenocortical cells increased P450scc mRNA accumulation (44). In NCI-H295R cells, $(Bu)_2$cAMP led to an increase in the level of P450scc mRNA at 12 h (45) and 20 h after treatment (46).

There was also no correlation between the levels of P450C21 mRNA and P450C21 protein in the ZG and ZFR.

TABLE 3. Effects of ACTH treatment for 9 days and dexamethasone (DEX) administration for 5 days on adrenal steroidogenic enzyme mRNAs

mRNA	Treatment	ZG	P^a	ZFR	P^a
StAR 1.6	CTR	1.00 ± 0.22		1.00 ± 0.11	
	ACTH	2.06 ± 0.26	*	8.65 ± 1.30	*
	DEX	0.40 ± 0.08	NS	4.49 ± 0.98	*
StAR 3.5	CTR	1.00 ± 0.20		1.00 ± 0.17	
	ACTH	2.69 ± 0.02	*	5.65 ± 0.29	*
	DEX	1.07 ± 0.14	NS	2.95 ± 0.69	*
P450scc	CTR	1.00 ± 0.14		1.00 ± 0.02	
	ACTH	0.90 ± 0.13	NS	2.60 ± 0.25	*
	DEX	0.24 ± 0.01	*	0.10 ± 0.003	*
P450C21	CTR	1.00 ± 0.09		1.00 ± 0.10	
	ACTH	0.79 ± 0.06	NS	1.02 ± 0.04	NS
	DEX	0.91 ± 0.12	NS	0.40 ± 0.08	*
3βHSD	CTR	1.00 ± 0.14		1.00 ± 0.03	
	ACTH	0.58 ± 0.05	*	0.88 ± 0.05	NS
	DEX	1.25 ± 0.15	NS	1.16 ± 0.04	*
P450C11	CTR			1.00 ± 0.06	
	ACTH			0.73 ± 0.21	NS
	DEX			0.02 ± 0.01	*
P450aldo	CTR	1.00 ± 0.05			
	ACTH	0.30 ± 0.03	*		
	DEX	1.39 ± 0.26	NS		
C-JUN	CTR	1.00 ± 0.20		1.00 ± 0.30	
	ACTH	0.18 ± 0.05	*	0.33 ± 0.08	NS
	DEX	0.98 ± 0.04	NS	1.06 ± 0.17	NS
AT_1	CTR	1.00 ± 0.25			
	ACTH	0.10 ± 0.01	*		
	DEX	1.18 ± 0.10	NS		

Control (CTR) values were arbitrarily fixed at 1.00. Data were expressed as the mean ± SEM (n = 3).

[a] Comparison between experimentals and controls: *, $P < 0.05$.

Indeed, we found no changes in mRNA levels, whereas the protein level was decreased during chronic ACTH treatment, as seen in Tables 3 and 4. *In vitro*, pharmacological doses of ACTH increased bovine adrenal P450C21 protein, whereas P450C21 mRNA increased only slightly (47).

In the adrenal ZFR of rats treated with ACTH for 9 days, the levels of P450C11 mRNA and protein were not significantly changed. These results are in agreement with those of Sander *et al.* (43), who reported that ACTH treatment of rats for 8 days did not stimulate P450C11 mRNA expression. However, Engeland *et al.* (48) reported that P450C11 increased in ACTH-treated rats between 1–4 days in the inner ZFR. Our results are in agreement with those of a previous study that reported that the level of P450C11 mRNA was not affected by a high plasma ACTH level induced by 3 days of 4-aminopyrazolopyrimidine treatment (49). Furthermore, ACTH administration for 24 h did not change the level of P450C11 mRNA or P450C11 protein in hamsters (23). *In vitro* in cultured bovine adrenocortical cells, ACTH increased the concentration of P450C11 transcripts (50).

In the case of 3βHSD, a decrease in mRNA was observed in the ZG but not in the ZFR, whereas no change in the protein level was found in either zone after chronic ACTH treatment. *In situ* hybridization studies performed on adrenals of rats treated with ACTH for 1–4 days showed that 3βHSD did not increase until 4 days (48). *In vitro* in NCI-H295R cells (51), protein kinase A signaling pathway activators enhanced, over a 48-h treatment period, the level of 3βHSD mRNA.

In this study, the P450aldo mRNA level in the rat adrenal ZG was decreased to 30% of the control value, showing that chronic ACTH administration also affected another rate-limiting enzyme of aldosterone formation. These results are in agreement with those of Sander *et al.* (43), which reported that 8 days of ACTH treatment rendered P450aldo mRNA undetectable in rat adrenals, and also with the results of Aguilera *et al.* (52), which reported that in isolated adrenal ZG cells from chronically stressed rats, P450aldo mRNA levels and binding of ^{125}I-[Sar^1,Ile^8]angiotensin II were significantly reduced. We reported that chronic ACTH administration to rats for 9 days resulted in a significant decrease in the capacity of adrenal ZG cells to bind angiotensin II (53). In agreement with our data, activation of the protein kinase A pathway in NCI-H295R cells rapidly decreased the level of AT_1 receptor mRNA, and this was paralleled by a loss of both AT_1 receptor binding and phosphoinositidase C response (54). The 90% decrease in AT_1 receptor mRNA level found in the ZG after chronic ACTH administration suggests that this change may be responsible for the decrease in the level of P450aldo mRNA. The bovine AT_1 receptor cDNA probe used recognizes both rat AT_{1A} and AT_{1B} receptors, and as the latter is the principal species in the rat adrenal (55), it is likely that the observed changes are due to changes in the level of AT_{1B} receptors.

In agreement with previous reports, we found that plasma corticosterone and aldosterone levels were increased during chronic ACTH stimulation for 9 days (53). A discrepancy thus seems to exist between the low adrenal contents of P450aldo and AT_1 receptor mRNA and the high plasma aldosterone level; at present we cannot explain this discrepancy. We can only speculate that 1) the antialdosterone antibody used may also have cross-reacted with unknown metabolites formed during chronic ACTH stimulation; and 2) it is possible that adrenal hypertrophy by itself might be responsible for the observed high plasma aldosterone level. Indeed, the rat adrenal increases in size during chronic ACTH administration (43, 53). The consequence of this hypertrophy is that the adrenal content of some steroidogenic enzymes might be considerably increased. For example, this is effectively the case for P450scc and P450C11. Indeed, when expressed per gland, P450scc protein content was significantly increased by 400% and 360% in the ZG and ZFR, respectively, and P450C11 protein content was significantly increased by 500% and 690% in the ZG and ZFR, respectively; hence, it is possible that the hypertrophy of the gland might have contributed to the formation of high concentrations of aldosterone precursors. As mentioned above, there was not always a correlation between changes in the level of mRNA and protein during chronic ACTH stimulation. This could be the consequence of a rapid turnover of steroidogenic enzyme mRNA and protein under such stressful conditions. A rapid turnover could also tentatively explain discrepancies found between results from different reports. Taken together, these results thus show profound perturbations in adrenal steroidogenic pathways in a situation similar to that of Cushing's

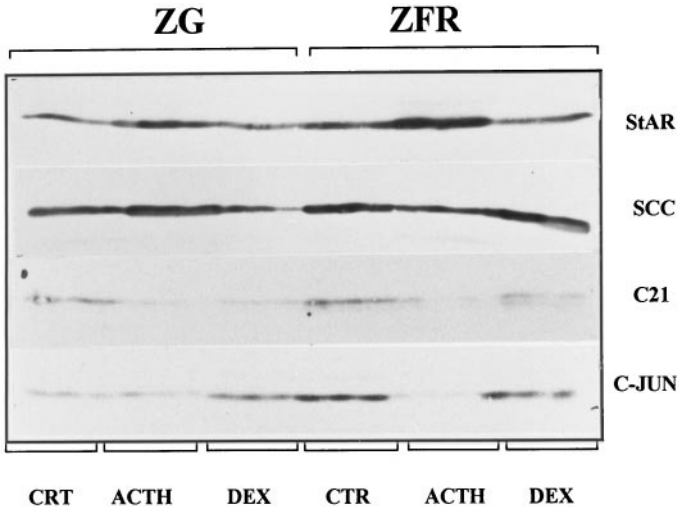

FIG. 7. Long-term effects of ACTH on rat adrenal proteins. Western blotting analyses on rat adrenal zona glomerulosa (ZG) and zonae fasciculata and reticularis (ZFR) containing the medulla. Three groups of male rats were injected twice daily for 9 days with the long acting ACTH preparation, and they were killed 12 h after the last injection; three additional groups received a daily injection of dexamethasone acetate (Dex) for 5 days, and they were killed 24 h after the last treatment. Three control groups were daily injected with 0.15 M NaCl (CTR). Analyses were performed on 50 μg of homogenate proteins. StAR, StAR protein; SCC, P450scc; C21, P450C21; C-JUN, c-*jun*.

syndrome, in which the adrenal is also under chronic ACTH stimulation.

When rats were injected twice daily to maintain a high plasma ACTH level, c-*fos* and *fos*B mRNAs were not detectable after 9 days of treatment. However, although decreased, c-*jun* and *jun*B mRNAs were still detectable under such conditions; moreover, c-*jun* protein, although decreased, was still detectable in the ZG and ZFR after 9 days of ACTH treatment. These results suggest a basic role for protooncogenes of the *jun* family in maintaining the integrity and function of the adrenal cortex. More work will be needed to delineate the function of protooncogenes in relation to the expression of StAR protein during chronic stimulation by ACTH.

Dexamethasone treatment for 5 days, to decrease ACTH secretion, decreased the level of circulating corticosterone and aldosterone and the levels of P450scc mRNA and P450C21 protein in the ZG. In the ZFR, the mRNA levels of P450scc, P450C21, and P450C11 were significantly decreased, whereas the levels of their respective proteins were not significantly changed by dexamethasone treatment. These results demonstrate the need for the presence of ACTH to maintain corticosteroidogenesis in a functional status. However, during dexamethasone treatment, the level of StAR mRNA was increased in the ZFR, but that of its protein remained unchanged. At present we cannot explain these differences; however, these results indicate that during low levels of circulating ACTH, the adrenals conserved a normal amount of StAR protein. Under such conditions, the level of c-*jun* mRNA was not different from control values in the ZFR. Although the relationship between protooncogenes of the Jun family and StAR protein in the ZFR has yet to be established, results obtained in this work open the way to further studies to clarify this point.

TABLE 4. Effects of long-term ACTH or dexamethasone (DEX) administration on different rat adrenal proteins, as analyzed by Western blotting

Protein	Treatment	ZG	P^a	ZFR	P^a
StAR protein	CTR	1.00 ± 0.01		1.00 ± 0.03	
	ACTH	1.48 ± 0.14	*	1.55 ± 0.04	*
	DEX	1.11 ± 0.05	NS	0.90 ± 0.03	NS
P450scc	CTR	1.00 ± 0.07		1.00 ± 0.15	
	ACTH	1.47 ± 0.08	*	0.56 ± 0.05	*
	DEX	0.63 ± 0.23	NS	1.03 ± 0.12	NS
P450C21	CTR	1.00 ± 0.01		1.00 ± 0.01	
	ACTH	0.27 ± 0.13	*	0.42 ± 0.09	*
	DEX	0.54 ± 0.04	*	0.72 ± 0.189	NS
3βHSD	CTR	1.00 ± 0.08		1.00 ± 0.08	
	ACTH	0.75 ± 0.10	NS	1.10 ± 0.01	NS
	DEX	0.78 ± 0.01	NS	0.97 ± 0.05	NS
c-*jun*	CTR	1.00 ± 0.06		1.00 ± 0.01	
	ACTH	0.84 ± 0.13	NS	0.25 ± 0.09	*
	DEX	1.91 ± 0.09	*	0.88 ± 0.08	NS

Control (CTR) values were arbitrarily fixed at 1.00. Data were expressed as the mean ± SEM (n = 3).

[a] Comparison between experimentals and controls: *, $P < 0.05$.

In conclusion, we have shown that acute stimulation by ACTH acts to increase the level of StAR mRNA, followed by a delay, by an increase in the level of StAR protein in the rat adrenal; this suggests that posttranscriptional modifications of the precursor of StAR protein occur before the translation of newly formed StAR mRNA. Our results suggest that during the first hour after ACTH stimulation, StAR protein acts on the external membrane of mitochondria. The rapid induction of protooncogenes of the *jun/fos* family also suggests their early participation in the action of ACTH to stimulate steroidogenesis.

Chronic stimulation by ACTH provoked the enlargement of the adrenal cortex and changes in the mRNA and protein levels of many steroidogenic enzymes of the ZG and ZFR. Under such conditions, the levels of StAR protein in both zones were elevated, showing the importance of this protein in the control of steroidogenesis during chronic ACTH stimulation, a situation that mimics Cushing's syndrome.

Acknowledgments

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