EFFECTS OF ACTH ON STEROID C-21 HYDROXYLATION IN RAT ADRENAL GLANDS

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SUMMARY

The activity of 21-hydroxylase in postmitochondrial fractions from normal and ACTH-stimulated rat adrenals has been measured using 11β-hydroxyprogesterone as substrate. Administration of 4 IU/day of long-acting ACTH for 4 days significantly increased basal levels of 21-hydroxylation without effect on NADPH-supported hydroxylation. Acute injection of ACTH, or prolonged administration of the hormone for 14–28 days did not have any effect on basal or NADPH-supported S.A. However, due to the increase in adrenal weight promoted by all the prolonged ACTH treatments, total enzyme activity will be raised in hypertrophic tissue. Adrenals from rats treated with ACTH for 4 days also showed [1] increased hydroxylation of 11β-hydroxyprogesterone after addition of glucose-6-phosphate to the incubations and [2] increased activity of the enzyme glucose-6-phosphate dehydrogenase. These results suggest that the enhanced 21-hydroxylation in 4 day ACTH-stimulated tissue may be the consequence of an activation of the hexose-monophosphate shunt.

INTRODUCTION

The hydroxylation of steroids at C-21 is a reaction which occurs in the endoplasmic reticulum of the adrenal cortex. The reaction requires NADPH and oxygen, as do other mixed function oxidases of steroid biosynthesis [1], and it appears to be specific for progesterone and its derivatives [2]. Among these, 11β-hydroxyprogesterone has been shown to be an efficient substrate for the 21-hydroxylase [3], and it has been suggested that this steroid may play a significant role in corticosterone biosynthesis [4, 5].

Previous biochemical and electron microscopic studies on adrenals from rats bearing an ACTH-secreting tumor, have suggested to us that this tissue might have an increased activity of the 21-hydroxylase [6, 7]. The effects of *in vivo* treatment of rats with ACTH on this hydroxylation step is the subject of the present report.

EXPERIMENTAL

Biological material

Mature female Wistar rats were used. The following treatments with ACTH were employed: "acute" ACTH: 50 IU of soluble ACTH i.p. 2 h before sacrifice; "subacute", 4 IU/day of long-acting ACTH sc for 4 days; "subchronic", 4 IU/day of long-acting ACTH sc for 14 days, and "chronic", 4 IU/day of long-acting ACTH sc during 21 days plus 8 IU/day for 8 more days. Control animals were left undisturbed. The day of the experiment the animals were killed by decapitation, and the adrenals were taken out, cleaned of fat and surrounding tissue and weighed on a torsion balance. Tissue was processed as described below.

Preparation of tissue fractions and incubation

Normal adrenal tissue ($\sim$80 mg) or an equivalent weight of tissue from ACTH-treated animals was homogenized in 1·3 ml of medium composed of 50 mM Tris buffer pH 7·4, 25 mM KCl, 5 mM MgCl$_2$ and 250 mM sucrose [8]. The homogenate was centrifuged in an International Equipment centrifuge at 900 *g* for 10 min at 4°C. The nuclear pellet was discarded and the mitochondria were sedimented at 14,500 *g* for 15 min: this treatment has been shown by Péron and Caldwell[9] to result in the sedimentation of heavy and light mitochondria. The postmitochondrial supernatant was purified by recentrifugation once more at 14,500 *g*. For assay of 21-hydroxylation in normal or ACTH-treated tissue, aliquots of the postmitochondrial supernatant were added to tubes containing 25 μg of 11β-hydroxyprogesterone as substrate [3] and 0·25 ml of the medium used for homogenization of the tissue. Total volume was kept at 0·5 ml. Incubations were carried out at 37°C for 30 min under air in a Dubnoff metabolic incubator with constant shaking.

Steroid determination

Steroids were extracted from the incubation medium with dichloromethane; the extracts were washed with 0·1 N NaOH and water, and an aliquot was used for the fluorimetric determination of corticosterone [7]. For this purpose, the dichloromethane extract was stirred with 2 ml sulfuric acid–ethanol (65:35 v/v), the mixture allowed to stand for 1 h at room temperature, and the fluorescence of the synthesized corticosterone was determined in an Aminco–Bowman spectrofluorometer. The contribution of endogenous fluorogenic steroids to the final corticos-

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terone values was determined by measuring the fluor-
escence of non-incubated samples (zero time values),
which was routinely subtracted from that of the incu-
bated samples. 11β-hydroxyprogesterone is not a
fluorogenic steroid. Protein was measured by the
method of Lowry et al.[10] and results of enzyme
activity expressed as μg corticosterone formed/mg
protein.

Measurement of glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase was deter-
mined in postmitochondrial supernatants from con-
trol rat adrenals and in adrenals from rats subjected
to a "subacute" ACTH treatment. The enzyme was
assayed according to the procedure of Harding and
Nelson[11], using NADP, 3.3×10^{-4} M, and glu-
cose-6-phosphate, 6.6×10^{-4} M. The rate of the reac-
tion was followed by measuring spectrophotometri-
cally the change in absorbancy at 340 nm at room
temperature. Enzyme activity was expressed as μmol
NADP reduced/min per mg protein.

Materials

The materials employed in this investigation were
obtained from the following sources: soluble ACTH
from Elea Laboratories (Argentina), long-acting
ACTH from Organon (Cortrofina-Z); NADPH (nico-
tinamide adenine dinucleotide phosphate, reduced
form), NADP (nicotinamide adenine dinucleotide
phosphate) and glucose-6-phosphate, from Sigma;
11β-hydroxyprogesterone from Mann Res. Labora-
tories. All other chemicals used were reagent grade.

RESULTS

Figure 1 shows the plot of 21-hydroxylase activity
(μg corticosterone formed/sample), versus variation in
the amount of protein per ml of incubation. The non-
linearity of the curve obtained is similar to that
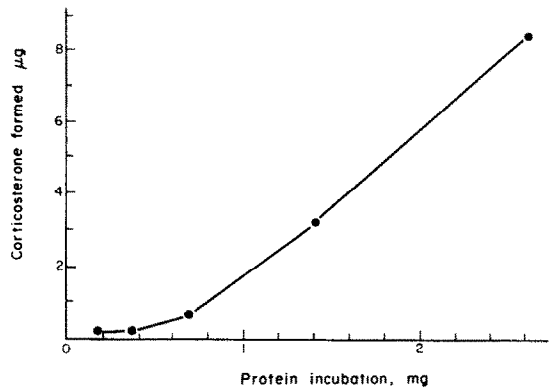reported for cholesterol side-chain cleavage [12] and

Fig. 1. 21-Hydroxylation of 11β-hydroxyprogesterone by
rat adrenal postmitochondrial supernatant. Enzyme acti-
vity is plotted versus increasing amounts of protein per
ml of incubation mixture. Assay of 21-hydroxylation con-
sisted of 50 mM Tris buffer pH 7.4, 25 mM KCl, 5 mM
$MgCl_2$, 250 mM sucrose and 50 μg 11β-hydroxyprogester-
one; total volume was 1.0 ml. After incubation for 30 min
at 37°C, the resulting corticosterone was extracted with
dichloromethane and measured by fluorimetry.

for 11β-hydroxylation [13], indicating that 21-hy-
droxylation is brought about by a complex enzyme
system similar to those involved in mitochondrial
hydroxylations.

The effect of several courses of ACTH injections
on this enzyme activity are presented in Table 1. The
conversion of 11β-hydroxyprogesterone to corticos-
terone was tested without additions, and after addi-
tion of 0.66 mM NADPH (500 μg/ml). The nucleotide
induced a significant increase in the reaction, which
was of the same magnitude in both control and
ACTH-treated preparations, for each treatment sche-
dule. The activity without additions, however, was
significantly elevated ($P < 0.01$) by treatment of rats
with 4 IU/day s.c. of long-acting ACTH for 4 days.
Neither a shorter stimulation, nor a more prolonged
treatment had a significant effect. Adrenal weight/100
g body weight, was increased two-fold after 4 days

Table 1. Effect of ACTH treatments in vivo on 21-hydroxylation of 11β-hydroxyprogesterone by rat adrenal post-
mitochondrial supernatants

Treatment	Adrenal weight (mg/100 B.W.)	21-hydroxylation (μg/corticosterone/mg protein)	
		no additions	NADPH (0.66 mM)
Control	27.6 ± 1.46 (n = 10)	0.21 ± 0.06 (n = 8)	1.40 ± 0.34 (n = 8)
Acute ACTH	24.2 ± 0.81 (n = 9)	0.43 ± 0.09 (n = 8)	1.30 ± 0.25 (n = 8)
Control	22.9 ± 1.29 (n = 12)	0.37 ± 0.08 (n = 8)	2.92 ± 0.25 (n = 8)
Subacute ACTH	42.5 ± 2.56 (n = 10)*	1.24 ± 0.25 (n = 8)†	3.39 ± 0.68 (n = 8)
Control	24.4 ± 1.23 (n = 12)	0.32 ± 0.09 (n = 8)	2.78 ± 0.39 (n = 8)
Subchronic	69.5 ± 4.72 (n = 8)*	0.58 ± 0.12 (n = 8)	2.85 ± 0.49 (n = 8)
Control	23.9 ± 1.47 (n = 6)	0.16 ± 0.06 (n = 4)	1.22 ± 0.27 (n = 4)
Chronic ACTH	118.4 ± 24.4 (n = 5)*	0.39 ± 0.14 (n = 4)	1.31 ± 0.43 (n = 4)

Acute ACTH: 50 IU of soluble ACTH i.p. 2 h before sacrifice; Subacute: 4 IU/day sc for 4 days; Subchronic:
4 IU/day for 14 days, and Chronic: 4 IU/day sc for 21 days plus 8 IU/day for 8 more days. Long-acting ACTH
was injected in the last three group treatments.
Results expressed as mean $\pm$ S.E.; n = number of observations.
* $p < 0.001$; † $p < 0.01$; all versus control rats.
Assay of 21-hydroxylation consisted of 50 mM Tris buffer pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose
and 25 μg 11β-hydroxyprogesterone; total volume was 0.5 ml. Total protein concentration (mg/ml) in the control
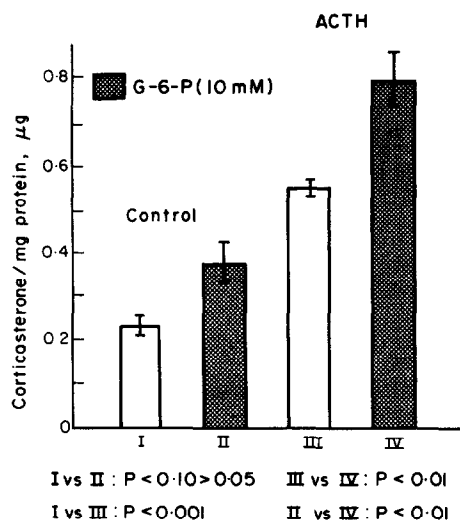supernatants was 1.3 ± 0.05, and in ACTH-treated adrenal supernatants was 1.3 ± 0.06.

ACTH

Fig. 2. Effects of ACTH treatment (4 IU/day for 4 days) on 21-hydroxylation of 11β-hydroxyprogesterone in the presence or absence of 10 mM glucose-6-phosphate. Assay of 21-hydroxylation as described in Table 1. Column I: basal levels in control rats; II: effect of glucose-6-phosphate in controls; III: basal levels in ACTH-treated rats; IV: effect of glucose-6-phosphate in ACTH-treated rats.

of ACTH, 3-fold after 14 days, and 6-fold with the "chronic" treatment.

Based on McKerns[14] observations, that ACTH activates glucose-6-phosphate dehydrogenase, we have investigated the effects of addition of glucose-6-phosphate to the incubations on the rate of 21-hydroxylation, and also have measured the activity of the dehydrogenase itself, in normal and ACTH-treated rat adrenals. Figure 2 shows that after 4 days of 4 IU/day of long-acting ACTH, the basal enzyme activity was increased, in confirmation with the results of Table 1. Glucose-6-phosphate, at 10 mM, significantly increased the hydroxylation rate in preparations derived from ACTH-treated animals, whereas only a borderline increase was detected with normal tissue. Furthermore, determination of S.A. of glucose-6-phosphate dehydrogenase in postmitochondrial supernatants from control and ACTH-treated rat adrenals, revealed that a similar treatment with the trophic hormone produced a significant increase in the levels of enzyme activity: the rate of reduction of NADP was 0.30 ± 0.01 μmol/min/mg protein in normal tissue (n = 4) and 0.41 ± 0.03 μmol/min/mg protein in adrenals derived from ACTH-treated rats ($n = 4$, $P < 0.025$).

DISCUSSION

Our results have shown that administration of 4 IU/day of long-acting ACTH for 4 days to female rats significantly increased the basal levels of 21-hydroxylation of 11β-hydroxyprogesterone. This treatment, however, was without effect on NADPH-supported hydroxylation. Furthermore, although adrenal weight increased according to the length of exposure of the animals to ACTH, the enzyme S.A. did not

parallel the overall increase in adrenal weight produced by the trophic hormone.

It is known that generation of cytoplasmic NADPH, the specific cofactor for 21-hydroxylation, occurs mostly through the hexose-monophosphate shunt, which is very active in the adrenal cortex [15]. According to McKerns[14], ACTH activates glucose-6-phosphate dehydrogenase, a key enzyme in this pathway, with an increased production of NADPH. The demonstration that the addition of glucose-6-phosphate resulted in increased 21-hydroxylation in ACTH-stimulated but not in normal tissue, lended some support to the work of McKerns. Further support for this hypothesis, however, was obtained by the observation that the enzyme glucose-6-phosphate dehydrogenase, measured in the postmitochondrial supernatants, showed higher activity levels in ACTH-treated adrenals, in comparison to the normal tissue. The evidences of Harding and Nelson[11] and Kuhn and Kissane[16], demonstrating that glucose-6-phosphate dehydrogenase is depressed after hypophysectomy or cortisone administration, are consistent with the possibility that the enzyme is under ACTH control. It is possible, therefore, that the increase in 21-hydroxylation after "subacute" ACTH treatment was due to an activation of the dehydrogenase enzymes of the pentose pathway. The time of exposure to ACTH is probably critical, since for some unknown reason, a more sustained treatment failed to increase the 21-hydroxylation. The evidence also supports the conclusion that the rate of production of NADPH was limiting in cytoplasmic steroidogenesis, since the addition of saturating concentrations of NADPH for reduction of the P-450 chain supported corticosterone production equally well in control and hypertrophied adrenals (Table 1). This was in contrast to the diminished response to NADPH by adrenal mitochondrion from "chronically" ACTH-treated rats, in studies on 11β-hydroxylation [17]; in this case, the electron carriers of the P-450 chain are probably impaired [18].

In spite of that the activity of 21-hydroxylase per mg protein was not modified after the "subchronic" and "chronic" treatments with the trophic hormone, total enzyme activity per gland will be raised, due to the concomitant adrenal hypertrophy. Thus, 21-hydroxylation in adrenal tissue from rats exposed to all ACTH treatments will be probably enhanced. This suggests that (a) more progesterone will be hydroxylated at C-21 to form deoxycorticosterone, with an increase in its secretory rate and in its intermediate role in steroid biosynthesis [6, 19, 20], and (b) if more 11β-hydroxyprogesterone is formed by ACTH-stimulated tissue [21, 22], this compound may become, at certain stage of ACTH stimulation, an alternative route for the biosynthesis of 21-hydroxysteroids.

Recently, we became aware of a report by Kittinger[23], in which this author demonstrated that ACTH enhances the conversion of 11β-hydroxyprogesterone to corticosterone in the adrenal glands of fetal Rhesus monkeys. This observation, as well as

ours, suggest that the effect of ACTH on 21-hydroxylation may be distributed in a wide variety of zoological species.

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