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THE EFFECTS OF CORTICOSTERONE, 18-OH-DOC, DOC AND 11β-HYDROXYPROGESTERONE ON THE ADRENAL PITUITARY AXIS OF THE STRESSED RAT

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SUMMARY

The *in vivo* effects of steroids native to the rat on the adrenocortical response to ether stress or to stress induced by a change of environment were studied in young male Sprague–Dawley rats. Corticosterone, 11β-hydroxyprogesterone and deoxycorticosterone at a dose of 1 mg per 100 g daily for 3 days, were effective to a similar degree in suppressing adrenal-pituitary function in the stressed rat as evidenced by a marked decrease in circulating corticosterone levels. Diminished adrenocortical activity in the steroid-treated animals was further indicated by a greatly reduced *in vitro* production of ultra-violet absorbing, Porter–Silber positive and fluorescent steroids and by a low corticosterone content in the gland. Under similar conditions 18-hydroxydeoxycorticosterone was either ineffective or slightly stimulated the adrenocortical stress response. Progesterone had no effect. The *in vitro* response of adrenal glands to ACTH was not impaired following steroid treatment, suggesting that the reduction in adrenocortical function observed not only with corticosterone but also with 11β-hydroxyprogesterone and deoxycorticosterone reflects a decrease in stress-induced ACTH secretion.

INTRODUCTION

Many investigators have shown that high levels of circulating corticosterone inhibit ACTH secretion in the rat, but currently there is not much emphasis on a similar role for mineralocorticoids. That these may be involved in negative feedback regulation of adrenal function has been indicated by the early observations that pharmacological doses of deoxycorticosterone acetate create symptoms of adrenocortical insufficiency [1], block stress-induced ACTH release as evidenced by adrenal ascorbic acid depletion tests [2], and cause histological changes in the zona glomerulosa of the hypophysectomized rat [3]. The role of 18-OH-DOC (18-hydroxy-deoxycort-costerone) on the adrenal-hypothalamo-pituitary axis has not been evaluated and is of interest because the production of this mineralocorticoid is under the control of ACTH [4–6] and may be implicated in the etiology of hypertension in both rat and man [7–10].

In this study the effects of *in vivo* administered corticosterone, 18-OH-DOC, DOC, 11β-hydroxyprogesterone and progesterone on adrenal function of the stressed rat have been compared.

EXPERIMENTAL

Male Sprague–Dawley rats, weighing 120-130 gm at the beginning of the experiment were grouped 4–5 per cage, and kept on a diet of standard Purina chow and water *ad lib*. They were weighed and injected s.c. daily at 10 a.m. with 0·1, 1 or 2 mg of steroid in 0·1 ml propylene-glycol per 100 g rat for 3 days. Control animals received an equivalent amount of propylene-glycol only, and one

group was handled and weighed but received no injections. Four h after the last injection, between 2 and 2.30 p.m., the animals were either exposed to ether vapours for 1 min. or transferred to a foreign environment. They were decapitated 5 min later.

Blood was collected in beakers, in some instances containing heparin, and centrifuged. Plasma or serum corticosterone levels were measured fluorometrically in a 65% sulfuric acid–alcohol mixture. The samples were processed and corrected for losses with radioactive tracer according to the method of Ganjam *et al.*[11], except that chromatographic isolation of corticosterone was not found to be essential and was therefore omitted. All fluorometric determinations were measured on an Aminco-Bowman spectrofluorometer using an activation wavelength of 465 mμ and an emission wavelength of 520 mμ.

Adrenals were rapidly removed, cleaned and weighed. They were then either immediately frozen, stored until a convenient time for analysis and then homogenized in 2 ml distilled water, or incubated intact in 2 ml Krebs–Ringer bicarbonate glucose buffer under an atmosphere of 95% O_2–5% CO_2 for 1 h periods. ACTH, when used, was added at a concentration of 3 units per 100 mg adrenal tissue to 2 ml of incubation medium containing either both or one adrenal gland.

The adrenal homogenates and incubation media were extracted three times with 2·0 ml methylene dichloride. The methylene dichloride extracts from adrenal homogenates were mixed with a tracer amount of [^{14}C]-corticosterone, to correct for losses, and subjected to paper chromatography in toluene-propylene-glycol [12]. The region containing corticosterone was then eluted with methanol and measured fluorometrically as above. Methylene dichloride extracts from incubation media were dried under nitrogen and redissolved in methanol. The 4-ene-3-ketone steroid fraction was measured by U.V. absorption at 240 nm on a Beckman DK2 recordiag spectrophotometer, corticosterone was measured by fluorometry and 18-OH-DOC by the Porter–Silber reaction[13]. The latter steroid was synthesized by Mr. Ming Li of this laboratory[14] or purchased from Searle de Mexico. 11β-Hydroxyprogesterone was obtained from Sigma Chemical Company, corticosterone and DOC from Mann Research Laboratories, and a clinical preparation of dexamethasone sodium phosphate, Decadron, from Merck, Sharp and Dohme. [^{14}C]-Corticosterone from New England Nuclear was used for studies involving radioactive tracer.

RESULTS

Effects of s.c. injected steroids on corticosterone levels in the stressed rat

Table 1 summarizes the effects of various steroids on the adrenocortical function of the stressed rat, subjected to a change of environment 5 min before decapitation. Plasma or serum levels of corticosterone, as well as the *in vitro* production of corticosterone during a 1 h incubation period were used as indices of adrenocortical activity. The use of propylene-glycol as an injection vehicle did not alter adrenocortical function. There was no evident change from control values following progesterone administration. Treatment with 18-OH-DOC also did not have a significant effect when the data from all the animals were pooled, although, as shown below (Table 2), in two out of the three experiments in which this steroid was used an increase of corticosteroid output over control values was observed. Corticosterone, 11β-hydroxyprogesterone and

Table 1. The effect of steroid treatment on corticosterone (B) levels in the stressed rat

Treatment	Dose (mg/100 g/day)	Production of B *in vitro* (μg/100 mg/h)	Concentration of B in Plasma (μg/100 ml)	Concentration of B in Serum (μg/100 ml)
Untreated		(4) 5·12±0·48		(6) 23·4±4·4
Control		(34) 4·40±0·64	(10) 21·9±2·8	(17) 25·7±1·6
Progesterone	1	(4) 4·52±0·75		(4) 28·5±2·6
Progesterone	2	(4) 5·04±1·36		
18-OH-DOC	1	(10) 4·96±0·63	(5) 24·6±5·6	(5) 19·7±3·4
DOC	1	(21) 2·24±0·25‡	(10) 7·3±1·9‡	(13) 7·6±1·5‡
DOC	2	(4) 1·56±0·15†		
11β-OH Progesterone	1	(4) 2·19±0·84*		(4) 11·7±4·3†
Corticosterone	0·1	(4) 3·99±0·65		
Corticosterone	1	(23) 1·29±0·12**	(9) 8·8±0·4‡	(13) 8·4±1·2‡
Corticosterone	2	(4) 1·17±0·36†		
Dexamethasone	0·1	(9) 1·38±0·18**		(7) 2·0±0·2‡

Rats were stressed by transfer to a new environment 5 min prior to decapitation. Steriods were dissolved in propylene glycol and administered in the dose indicated. Control animals were injected with vehicle only. Untreated animals were handled but not injected. Numbers in brackets represent the number of rats in each group and values shown are means ± S.D.M. Significance of difference from control group: $*P < 0{\cdot}05$; $\dagger P < 0{\cdot}01$; $\ddagger P < 0{\cdot}001$ (t-tests).

DOC, at a dose of 1 mg per 100 g, inhibited adrenocortical function as evidenced by a much reduced *in vitro* production and greatly diminished circulating levels of corticosterone. Dexamethasone, at 0·1 mg per day, was also a potent inhibitor of the stress response although the same amount of corticosterone had little or no effect.

Table 2. Steroid treatment and adrenocortical activity of the stressed rat

Expt.	Treatment		Steroid production *in vitro* (μg/100 mg/h) U.V.	PS	FL	Adrenal B (μg/100 mg) FL	Plasma B (μg/100 ml) FL
I	Control	(4)				3·44±0·89	
	18-OH-DOC[a]	(4)				5·33±1·02	
	DOC	(4)				0·26±0·02†	
	Corticosterone	(4)				0·46±0·18†	
II	Control	(5)	7·79±1·31	1·91±0·28	3·76±0·75		16·2±3·4
	18-OH-DOC[b]	(5)	11·32±0·82*	2·68±0·10*	5·98±0·96*		24·6±5·6
	DOC	(5)	3·42±1·07†	0·99±0·29†	2·17±0·57		6·6±2·4
	Corticosterone	(5)	4·19±0·86*	0·91±0·09†	1·65±0·22*		8·1±0·5
III	Control	(5)	15·91±1·34		4·67±0·30		26·9±4·2
	18-OH-DOC[b]	(5)	14·92±2·26		3·93±0·58		19·7±3·4
	DOC	(2)	7·83±0·70*		2·05±0·39†		3·9±0·8†
	Corticosterone	(2)	9·16±2·55		1·66±0·42†		3·2±0·2†

The animals were stressed by transfer to a new environment 5 min prior to decapitation. Steroids were administered at a dose of 1 mg per 0·1 ml propylene-glycol per 100 g. Control animals were injected with propylene-glycol only. Numbers in brackets indicate the number of animals per group. Values shown are means ±S.D.M. The analyses of variance indicated highly significant differences associated with treatment means in all cases (P values $< 0{\cdot}005$ and $< 0{\cdot}01$) excepting the U.V. determinations of Expt III ($0{\cdot}05 < p < 0{\cdot}1$). Differences from controls were assessed by the LSD test and significant differences are indicated by asterisks: $*P < 0{\cdot}05$; $\dagger P < 0{\cdot}01$.

Comparison of plasma corticosterone levels with corticosterone output in vitro

Figure 1 illustrates the close parallelism between the circulating corticosterone levels at time of death and the *in vitro* corticosterone production during a 1 h incubation period immediately following decapitation. Similar comparisons between *in vitro* and *in vivo* indices of adrenocortical function have been made in other laboratories [15, 16]. It is also apparent that exposure to ether vapours for a 1 min period created more stress in control rats than the transfer to a new location. However, ether stress did not overcome the block in pituitary-adrenal function found in steroid pretreated animals.

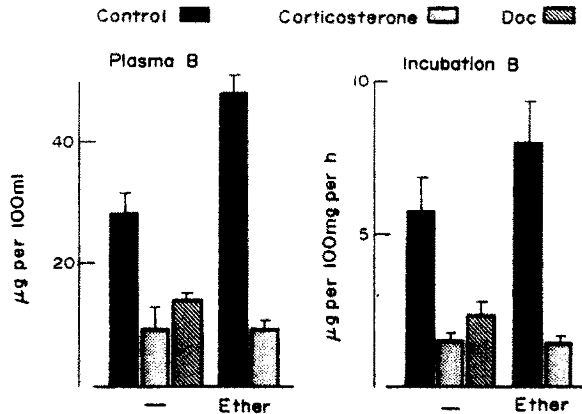

Fig. 1. The effects of corticosterone (B) and of DOC (1 mg/100 g daily for 3 days) on the adrenocortical stress response as measured by plasma B levels and B production *in vitro*. Rats, 4 per group, were stressed either by transfer to a new environment or by exposure to ether vapours for 1 min and decapitated 5 min later. The vertical lines above the bars indicate standard errors of the mean.

Effects of s.c. injected 18-OH-DOC, DOC *and corticosterone on plasma and adrenal corticosterone content and on the* in vitro *formation of ultra-violet absorbing (U.V.), Porter–Silber positive (PS) and fluorescent (FL) steroids*

In Table 2 the effect of 18-OH-DOC on the adrenocortical function of the stressed rat is presented in greater detail and compared to that of DOC and of corticosterone during the same experimental conditions. Adrenal corticosterone concentrations at the time of death are shown in experiment I. Corticosterone levels in glands from both DOC and corticosterone pretreated animals were reduced to about 10% of those found in control glands, whereas a tendency towards an increase over control values was found in adrenals from animals which had been treated with the same amount of 18-OH-DOC. Experiment II shows plasma corticosterone levels and *in vitro* corticoid production during the 1 h incubation period immediately after decapitation. In addition to a drop in *in vitro* production and in plasma concentration of corticosterone following injections of either DOC or corticosterone a diminution of adrenocortical activity was further substantiated by a low *in vitro* output of 18-OH-DOC, and by a decrease in total 4-ene-3-ketonic steroids. In contrast, the adrenocortical function in animals pretreated with 18-OH-DOC was greater than that in controls as is evidenced by an increase in all fractions measured. It should perhaps be noted, that adrenocortical activity in the propylene-glycol injected stressed control animals was lower than usual in this experiment. There was no indication for a

negative feedback role of 18-OH-DOC on its own secretion as may be seen by a relatively high *in vitro* production of Porter–Silber positive material by adrenals from 18-OH-DOC treated rats. In experiment III treatment with 18-OH-DOC did not affect the *in vitro* production of ultraviolet absorbing steroids, but resulted in a slight, although not significant, decrease in serum levels and in the *in vitro* output of corticosterone. This study differed from the others in that the animals were kept in isolation and were possibly subjected to greater stress during the three day injection period prior to sacrifice.

In vitro *response to ACTH of adrenals from steroid treated rats*

In order to see whether the observed changes in adrenocortical function following steroid adminstration are related to differences in circulating ACTH levels or due to some defect in the adrenal gland itself, the *in vitro* response to ACTH of adrenals from steroid treated and control animals was compared.

In Fig. 2 both adrenals from each rat were incubated for 4 h with a change of medium after each h. ACTH from Sigma, 3 units per 100 mg tissue, was added to the medium after the second incubation period. The bar graph on the right shows the *in vitro* production of corticosterone in control adrenals, before and after the addition of ACTH, and, the figure on the left depicts the per cent change

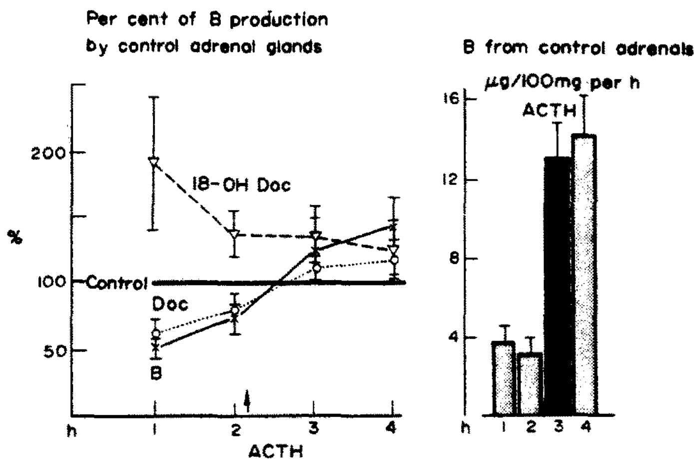

Fig. 2. The *in vitro* response of adrenals from steroid-treated animals to ACTH. Both glands were incubated in 2 ml KRBG for 4 h with a change of medium after each h. ACTH, 3 units per 100 mg, was added during the 3rd h. The effect of steroid treatment (1 mg/100 g daily for 3 days) on corticosterone (B) production *in vitro*, expressed as per cent of control values, is shown on the left; B production by control adrenals, on the right. Values represent the means of 5 animals and the vertical lines indicate standard errors of the mean.

in corticosterone production from control values by adrenal tissues from rats treated with corticosteroids. The animals were killed in groups of 4 over a $2\frac{1}{2}$ h period and the *in vitro* corticosterone production in the control animal of each group was taken to be 100%. As previously shown, corticosterone production was reduced during the first one hour incubation period in adrenals from both corticosterone and DOC treated rats but was elevated in those from 18-OH-DOC treated animals. These effects were not so marked during the second incubation

period and approached control values. Addition of ACTH during the 3rd h of incubation stimulated corticosterone formation 4–10 fold in all samples indicating that, at least *in vitro*, the adrenocortical response to ACTH was neither stimulated nor impaired by steroid treatment.

It is, however, conceivable that after a 2 h incubation period initial differences may no longer be present. The effect of ACTH on *in vitro* corticosterone output during the immediate incubation period following decapitation was therefore studied. In these studies one adrenal was stimulated with ACTH, in the same concentration as used earlier, while the other served as a control. The results are summarized in Table 3. ACTH stimulated corticosterone production to a similar degree in adrenals from both steroid-treated and control animals. However in contrast with the previous study only a two-fold increase in corticosterone output could be observed. It is well known that the adrenal cortex is relatively insensitive to *in vitro* stimulation by exogenous ACTH during initial incubation stages [17].

Effects of s.c. injected steroids on thymus and adrenal weights

In Fig. 3 the thymus weights of steroid-treated animals are compared to those of control rats. A dramatic involution of the gland occurred following treatment with 0·1 mg of dexamethasone. At a dose of 1 mg per day a significant decrease in thymus weights was also found with corticosterone ($P < 0{\cdot}001$) and 11β-hydroxyprogesterone ($P < 0{\cdot}01$) and, to a lesser degree, with 18-OH-DOC ($P < 0{\cdot}05$). Progesterone and DOC had no effect.

Except for a slight decrease following the administration of either 0·1 mg of dexamethasone or 2 mg of corticosterone there was no evident change in adrenal weights.

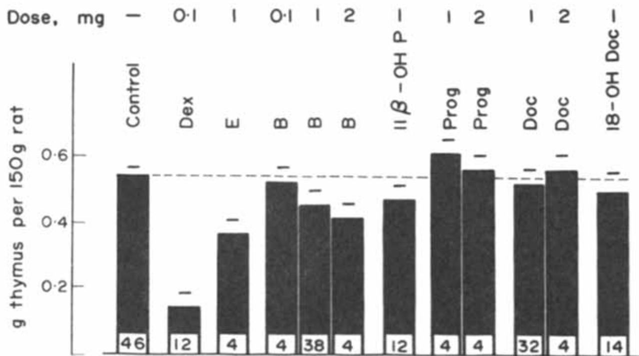

Fig. 3. The effects of steroid treatment on thymus weights. Steroids were dissolved in propylene-glycol and injected daily for 3 days in the dose indicated per 100 g rat. The horizontal lines above the bars indicate standard errors of the mean. The numbers at the base of the bars represent the number of animals in each group.

DISCUSSION

Although it has been recognized for some time that DOC blocks the secretion of pituitary ACTH [1, 2], and that this effect is mediated via the central nervous system (CNS) [18], current investigations on the negative feedback action of adrenal steroids on the CNS-pituitary-adrenal axis concentrate primarily on glucocorticoids either native or foreign to the species or synthetic. The present

Table 3. *In vitro* response of adrenals from steroid-treated rats to ACTH

		μg corticosterone per 100 mg adrenal tissue/h						
Expt.	ACTH	Control	18-OH-DOC (1mg/100 g)	DOC (1 mg/100 g)	Prog (1 mg/100 g)	11β-OH-P (1 mg/100 g)	B (1 mg/100 g)	Dex (0·1 mg/100 g)
I		5·33 ± 0·56*		1·91 ± 0·40			1·06 ± 0·13	
	+	8·12 ± 1·16		5·32 ± 1·60			2·72 ± 0·48	
II		4·28 ± 0·40		2·84 ± 0·97	4·52 ± 0·75	2·19 ± 0·84	1·23 ± 0·33	
	+	8·41 ± 1·10		5·06 ± 1·27	6·64 ± 0·84	4·95 ± 1·22	2·84 ± 0·32	
III		4·67 ± 0·30′	3·93 ± 0·58″	2·05 ± 0·39″			1·66 ± 0·42′	1·64 ± 0·58′
	+	11·09 ± 0·63	9·56 ± 1·02	5·07 ± 0·54			5·58 ± 1·54	7·22 ± 0·61

In expts. I and II the animals, 4 per group, were stressed by exposure to ether vapours for 1 min and killed 5 min later.

In expt. III animals were stressed by transfer to a new environment 5 min prior to sacrifice, ′2 rats per group; ″5 rats per group.

ACTH (3 units per 100 mg) was added to one adrenal; the other incubated concurrently served as endogenous control.

*Values represent means ± S.D.M.

study indicates that not only corticosterone but also DOC as well as 11β-hydroxyprogesterone inhibit effectively the CNS-pituitary-adrenal axis of the stressed rat. During stress there is an increased secretion of corticosterone and of DOC in the rat and, since there is evidence for the endogenous production of 11β-hydroxyprogesterone as well, it is possible that the three, corticosterone, DOC and 11β-hydroxyprogesterone, act in tandem to inhibit the secretion of ACTH. A synergistic action of several hormones may partly explain the difficulty to find effective negative feedback regulation by so-called physiological increases in endogenous levels of only one single hormone [19, 20]. Although 11β-hydroxyprogesterone is only a minor component of adrenocortical secretion [21], the production of DOC is normally about 1/10th that of corticosterone in the rat [22, 23]. Under certain pathological conditions, however, such as adrenal enucleation [10], injections of Metopirone [24] or implantation with a mammotropic tumor [25], the secretory rates of DOC under normal surgical stress may approach those of 18-OH-DOC and corticosterone in some animals. A surge of endogenous DOC could explain the biphasic rise in plasma ACTH following Metopirone administration seen in the rat [26]. In man, also, the secretion of DOC is stimulated by ACTH [27, 28] and plasma levels of DOC are considerably raised in some forms of hypertension [29, 30] and following treatment with Metopirone [31].

Both DOC and 11β-hydroxyprogesterone are immediate precursors of corticosterone and the possibility that their *in vivo* effects be mediated via the prior synthesis of corticosterone must be considered. However, *in vivo* conversion to corticosterone would perhaps not be expected to exceed 10% and under the present experimental conditions 0·1 mg corticosterone did not effectively block adrenocortical function. Furthermore another precursor, progesterone, was ineffective and in fact has been reported to increase corticosterone levels *in vivo* [32, 33].

In contrast to the inhibitory effects of corticosterone, DOC and 11β-hydroxyprogesterone on adrenocortical function during stress, 18-OH-DOC was either ineffective or tended to stimulate adrenocortical activity. It does not therefore appear to be directly involved in negative feedback regulation of the CNS-pituitary-adrenal axis, although, in the stressed rat, its production may equal or even exceed that of corticosterone [10]. Selective inhibition of its own production at the adrenal level could result in a shift of endogenous precursor towards corticosterone synthesis and thereby explain the increase observed in plasma and adrenal corticosterone which in turn could account for the decrease observed in thymus weights in 18-OH-DOC treated rats. The increased *in vitro* output of the Porter-Silber chromogen paralleled by a proportionally higher output of corticosterone (Table 2) however, argues against this and rather suggests a secretory pattern of ACTH-stimulated adrenal tissue. Although the administered dose could fall within the physiological range of a stressed rat, assuming that absorption may not be complete, the hormone itself may be a stressful agent. If this were so, an increase in adrenocortical activity over endogenous resting levels could be expected in 18-OH-DOC treated animals.

It should be noted that adrenocortical activity was assessed during an early phase of the stress response, 5–7 min after initiation of the stress. Another explanation for the enhanced steroid production observed in 18-OH-DOC treated animals could therefore be an increased sensitivity of the CNS-pituitary-adrenal axis to stress, marked by a more rapid response than in control animals. Maximal

rates of *in vitro* corticosterone production are reported to occur 15 min [16] and peak levels of plasma corticosterone 30 min after stress [35]. An early stimulatory effect of 18-OH-DOC would then not be masked by an already maximally stimulated CNS-pituitary-adrenal axis. The possibility that 18-OH-DOC exerts a positive feedback action on the CNS-pituitary-adrenal axis to counterbalance the negative effects of corticosterone and DOC is worthy of further investigation.

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