Journal of Steroid Biochemistry, 1971, Vol 2, pp. 237–244. Pergamon Press. Printed in Great Britain

EFFECT OF ANDROGENS ON ADRENAL CORTICAL 11β-HYDROXYLATION

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(*Received* 2 *March* 1971)

SUMMARY

The effects of the chronic administration of testosterone, androstenedione and DHA upon adrenal cortical function in the rat were compared. Only testosterone induced hypertensive disease and decreased 11β- and 18-hydroxylase activity *in vitro*. Significant decreases in adrenal mitochondrial cytochrome P-450 levels were apparent in all androgen-treated groups. Testosterone administration also resulted in a significant increment in the concentration of cytochromes $a + a_3$. The DOC-induced difference spectrum was reduced in all androgen-treated groups when expressed per mg of protein but only in mitochondria from testosterone-treated rats when calculated per nanomole of cytochrome P-450. The results suggest that the inhibition of the 11β- and 18-hydroxylation of DOC by testosterone is the result of competitive binding to cytochrome P-450 and not the induced decrease in cytochrome P-450 levels *per se*.

INTRODUCTION

THE role of the gonadal hormones in the regulation of adrenocortical function in the rat is well documented[1, 2]. Reports from this laboratory have revealed that the administration of large doses of testosterone[3] or synthetic androgenic compounds[4, 5] to properly sensitized rats may so modify their adrenocortical secretory pattern that hypertensive cardiovascular disease results. More recent studies[6, 7] have been concerned with the pathogenesis of this androgen-induced hypertension. The present communication compares the effects of the three principal naturally-occurring androgens upon steroidogenesis by the rat adrenal cortex and the resulting blood pressure changes.

MATERIALS AND METHODS

Female, 35-day-old Sprague–Dawley rats obtained from the Holtzman Co. were used. Females were employed because of their greater susceptibility to the development of hypertension. They were individually caged and maintained in a room with 12 h light and dark cycles at a constant temperature of 22 ± 1°C. Rats were fed Purina Lab Chow *ad libitum* and given tap water as drinking solution for one week at which time right nephrectomy was performed on all animals. Thereafter all rats were given 1% sodium chloride as drinking solution *ad libitum*. Treatment was initiated 3 days following uninephrectomy.

All androgens were obtained from Steraloids, Inc. and checked for purity by melting point determination and thin layer chromatography on silica gel. Experimental rats received 10 mg of testosterone, DHA* or androstenedione daily for

*Trivial names and abbreviations used: Corticosterone (B): 11β,21-dihydroxy-4-pregnene-3,20-dione; dehydroepiandrosterone (DHA): 3β-hydroxy-5-androsten-17-one; androstenedione: 4-androstene-3,17-dione; 11-deoxycorticosterone (DOC): 21-hydroxy-4-pregnene-3,20-dione; 18-hydroxydeoxycorticosterone (18-OH-DOC): 18,21-dihydroxy-4-pregnene-3,20-dione.

35 days by subcutaneous injection of a microcrystalline suspension in 0·2 ml of corn oil. Controls received the vehicle alone for the same length of time. Rats were sacrificed 24 h after the final injection. All rats were weighed weekly and indirect systolic blood pressure measured on the tail using a Physiograph Four (E and M Instrument Co., Houston, Texas) with the rats under light ether anesthesia.

At the end of the treatment period all rats were killed by decapitation. Adrenals were rapidly removed, carefully freed of adhering fat, weighed and pooled by group. Homogenates were prepared in 0·25 M sucrose–0·01 M Tris (pH 7·4) with a Thomas tissue grinder consisting of a Teflon pestle and grinding vessel made of borosilicate glass. Appropriate volumes of buffer were added so that the final homogenate concentration was 35 mg/1·0 ml. One ml of the homogenate suspension was incubated with 0·1 μCi [4-^{14}C] progesterone (S.A. 4·0 mCi/mmol) and 4·0 ml of incubation buffer (0·1 M Tris-chloride, 0·005 M KCl, 0·005 M $MgCl_2$, 0·08 M NaCl, 0·05 M sucrose, pH 7·4) containing sodium malate (100 mg per 100 ml). Incubations were carried out in duplicate for 10 min at 37° in air. Steroids were extracted from the incubation medium with methylene chloride and steroid analyses performed as previously described[8].

The residual homogenate preparations were centrifuged at $700 \times g$ for 10 min to remove nuclei, red cells and unbroken cells and the supernatant centrifuged at $10{,}000 \times g$ for 10 min to obtain the mitochondrial fraction. Mitochondria were washed once with sucrose-Tris chloride buffer and recentrifuged at $10{,}000 \times g$ for 10 min. Mitochondria were then suspended in the same buffer at a concentration of mitochondria equivalent to 50 mg tissue/ml (approx. 1·5 mg protein per ml).

For mitochondrial incubations with radioactive DOC. 1·0 ml of mitochondrial suspension was added with 0·15 μCi of [4-^{14}C] DOC (S.A. 0·65 mCi/mmol) in 0·1 ml ethanol to 4 ml of incubation buffer containing 100 mg of isocitrate per 100 ml. Incubations and steroid analyses were performed as for the homogenates.

The cytochrome concentrations in adrenal mitochondrial preparations were determined by the methods of Cammer and Estabrook[9] using an Aminco–Chance dual wavelength scanning recording spectrophotometer. Substrate-induced spectral changes[10] were also evaluated by techniques previously reported using this same instrument. Protein assays were performed by the method of Lowry *et al.*[11].

RESULTS

Body weights and blood pressures

At the end of the treatment period, the mean body weights of the four groups did not differ significantly although the androstenedione-treated rats were somewhat larger than the rest (Table 1).

The testosterone-treated group showed a significant elevation in systolic blood pressure as compared with controls (172 ± 5 mm Hg vs. 123 ± 5 mm Hg) (Table 1). The rats treated with androstenedione and DHA also had greater mean blood pressures (135 ± 7 mm Hg and 133 ± 5 mm Hg respectively) than controls, but those differences were not statistically significant.

Organ weights

All three androgens produced significant adrenal atrophy with androstenedione

Table 1. Body weights, organ weights and systolic blood pressures of control and androgen-treated rats

Group and treatment	Number of rats	Body weight (g)		Blood pressures (mm Hg)		Organ weights			
		Initial	Final	Initial	Final	Kidney (mg)	Heart (mg)	Pituitary (mg)	Adrenals (mg)
Control	15	159 ± 3*	238 ± 5	96 ± 2	123 ± 5	1550 ± 40	940 ± 30	13·3 ± 0·6	68·4 ± 1·8
Testosterone	21	157 ± 3	238 ± 6	95 ± 3	172 ± 5	2790 ± 80	1240 ± 30	10·6 ± 0·3	57·1 ± 1·1
Androstenedione	21	160 ± 4	254 ± 6	98 ± 2	136 ± 7	2550 ± 80	1140 ± 40	13·3 ± 0·4	47·3 ± 2·1
DHA	21	157 ± 3	237 ± 4	96 ± 2	133 ± 5	2220 ± 60	1130 ± 30	14·9 ± 0·5	52·4 ± 1·6

*Standard error of the mean.
——— $p < 0{\cdot}01$ (vs. controls).

inducing the greatest decrease in size (Table 1). There was also marked cardiac hypertrophy and a significant increase in kidney size in the androgen-treated groups. Testosterone caused the greatest increase in the size of these two organs, as would be expected based upon the respective mean blood pressures. Only testosterone administration produced significant atrophy of the pituitary gland.

Adrenal homogenate incubations

Table 2 shows the results of the incubation of adrenal homogenates, obtained from control and androgen-treated rats, with [4-^{14}C] progesterone. The progesterone was quite readily metabolized in all the incubation flasks, as very little was recovered at the end of the incubation period. No significant differences existed between control and androgen-treated groups in the amounts of progesterone metabolized. There was, however, considerable variation in the amounts of the various metabolites in which the radioactivity was recovered. Controls converted almost all of the progesterone to corticosterone and 18-OH-DOC, the main secretory products of the rat adrenal cortex, and very little DOC was recovered from the incubation medium. The homogenates from the testosterone-treated rats, on the other hand, were able to effectively metabolize the progesterone only as far as DOC, with very little conversion to more polar steroids. Thus, testosterone administration resulted in a marked reduction in adrenal 11β- and 18-hydroxylase activities. The homogenates obtained from DHA and androstenedione-treated rats converted considerably more of the progesterone to corticosterone and 18-OH-DOC than did those from testosterone-treated rats. However, even for these two groups, significantly more DOC and less corticosterone was recovered from the incubation medium than from controls.

Mitochondrial incubations

In order to evaluate the effects of androgen treatment upon adrenal 11β- and 18-hydroxylation more directly, adrenal mitochondria from the various groups were isolated and incubated with [4-^{14}C] DOC. The results are presented in Table 3. It can be seen that mitochondria from controls converted most of the DOC to corticosterone and 18-OH-DOC. Less than 20% of the radioactivity

Table 2. *In vitro* metabolism of [4-^{14}C] progesterone by adrenal homogenates from control and androgen-treated rats

Group and treatment	Number of experiments	Percent conversion of [4-^{14}C] progesterone to:			
		Progesterone*	DOC	B	18-OH-DOC
Control	4	7·8 ± 1·8†	5·5 ± 0·9	62·3 ± 4·8	26·2 ± 2·2
Testerosterone	4	5·1 ± 1·0	67·4 ± 5·2	11·2 ± 1·2	5·7 ± 0·8
Androstenedione	4	4·2 ± 1·4	27·4 ± 4·3	40·2 ± 3·2	20·6 ± 3·2
DHA	4	5·3 ± 1·3	28·7 ± 3·9	38·4 ± 3·8	17·9 ± 2·1

*Unmetabolized progesterone.
†Standard error of the mean.
———— $p < 0{\cdot}01$ (vs. controls).
– – – – – – $p < 0{\cdot}05$ (vs. controls).

Table 3. *In vitro* metabolism [4-^{14}C] DOC by adrenal mitochondria from control and androgen-treated rats

Group and treatment	Number of experiments	Mitochondrial protein (mg/ml)	Percent conversion of[4-^{14}C] DOC to:		
			DOC*	B	18-OH-DOC
Control	4	1·62 ± 0·21†	15·8 ± 2·1	50·2 ± 4·3	22·7 ± 2·1
Testosterone	4	1·67 ± 0·28	59·5 ± 4·3	23·8 ± 2·1	11·1 ± 1·9
Androstenedione	4	1·53 ± 0·19	15·5 ± 2·1	47·6 ± 3·8	23·2 ± 2·2
DHA	4	1·65 ± 0·31	14·3 ± 1·8	50·6 ± 3·4	23·7 ± 1·8

*Unmetabolized DOC.
†Standard error of the mean.
——— $p < 0·01$ (vs. controls).

was recovered as residual DOC. The metabolism by mitochondria from androstenedione- or DHA-treated rats was essentially the same as that of controls, with no detectable impairment in mitochondrial hydroxylating activity. The mitochondria from those rats treated with testosterone, on the other hand, were able to convert less than half of the added DOC to more polar compounds, consistent with marked inhibition of 11β- and 18-hydroxylation.

Adrenal cytochrome concentrations

Adrenal mitochondrial cytochrome levels in control and androgen-treated rats are reported in Table 4.

The concentrations of cytochromes b and $c + c_1$ were not affected by treatment with any of the androgens. The $a + a_3$ levels in the adrenals of testosterone-treated rats were, however, more than double those in controls. The administration of DHA and androstenedione produced small but insignificant increases in the concentration of these cytochromes.

The levels of mitochondrial cytochrome P-450, the terminal oxidase for 11β- and 18-hydroxylation[12, 13], were significantly reduced in all of the androgen-treated groups. The greatest decrease was observed in the adrenals from the testosterone-treated rats. We have previously[6] shown this apparent reduction in cytochrome P-450 to be real, and not the result of androgenic interference with its accurate assay, by the demonstration of a comparable decrease in total mitochondrial protoheme.

Table 4. Adrenal mitochondrial cytochrome concentrations in control and androgen-treated rats

Group and treatment	Number of experiments	Cytochrome concentration (η moles/mg Protein)			
		$a + a_3$	b	$c + c_1$	P-450
Control	4	0·04 ± 0·01†	0·13 ± 0·02	0·19 ± 0·02	2·10 ± 0·20
Testosterone	4	0·10 ± 0·02	0·12 ± 0·01	0·20 ± 0·03	1·01 ± 0·15
Androstenedione	4	0·06 ± 0·01	0·13 ± 0·02	0·21 ± 0·02	1·29 ± 0·21
DHA	4	0·06 ± 0·01	0·14 ± 0·03	0·21 ± 0·03	1·18 ± 0·17

†Standard error of the mean.
——— $p < 0·01$ (vs. controls).

DOC-induced spectral changes

It has previously been reported that the addition of DOC to bovine[10] or rat[7] adrenocortical mitochondria produces a difference spectrum with an absorption maximum at 385 nm and a minimum at 420 nm.

Adrenal mitochondria isolated from control and androgen-treated rats were titrated with DOC. The change in optical density at 385 nm minus that at 420 nm was calculated for each addition of DOC (Table 5).

It is apparent that the magnitude of the spectral change induced by DOC was considerably smaller in adrenal mitochondria from the androgen-treated rats than in those from controls. The smallest response was acquired from those mitochondria from testosterone-treated rats, less than one third that of controls.

Since the magnitude of the substrate-induced spectral change depends upon the concentration of cytochrome P-450 and since androgen treatment produced marked decreases in the levels of this cytochrome, the induced difference spectrum in each group was also expressed per nanomole of cytochrome P-450 (Table 5). Even after this correction for differences in P-450 concentrations, the DOC-induced spectral change in the mitochondria from testosterone-treated rats was still less than half that in controls. However, the apparent differences in the magnitude of the spectra from androstenedione- and DHA-treated rats and controls were eliminated after this reevaluation. Therefore, the capacity of DOC to interact with cytochrome P-450 was reduced only after testosterone treatment.

Table 5. DOC-induced spectral changes in adrenal mitochondria from control and androgen-treated rats

Group and treatment	Cytochrome P-450 (η moles/mg Prot)	DOC concentration (μM)	$\Delta A_{385-420}$ ($\times 10^{-2}$)	$\Delta A_{385-420}/\eta$ mole P-450 ($\times 10^{-2}$)
Control	2·10	1·0	3·20 ± 0·40†	0·94 ± 0·10
		2·5	5·90 ± 0·79	1·73 ± 0·22
		4·0	7·39 ± 0·83	2·17 ± 0·26
Testosterone	1·01	1·0	0·91 ± 0·11	0·54 ± 0·09
		2·5	1·55 ± 0·23	0·92 ± 0·15
		4·0	1·80 ± 0·22	1·07 ± 0·11
Androstenedione	1·29	1·0	1·95 ± 0·17	0·99 ± 0·08
		2·5	3·35 ± 0·42	1·70 ± 0·19
		4·0	4·20 ± 0·40	2·13 ± 0·20
DHA	1·18	1·0	1·77 ± 0·29	0·91 ± 0·07
		2·5	3·21 ± 0·35	1·65 ± 0·17
		4·0	4·03 ± 0·39	2·07 ± 0·21

†Standard error of the mean.
———— $p < 0·01$.

DISCUSSION

It is well established that several androgenic compounds, when administered to properly sensitized rats, induce hypertensive cardiovascular disease[2, 3, 5]. The preponderance of evidence available implicates adrenocortical dysfunction in the pathogenesis of these models of experimental hypertension.

The results of previous studies from this laboratory[2, 3] have supported the hypothesis that the specific biochemical defect resulting from androgen administration is impaired adrenocortical 11β- and 18-hydroxylation, causing an increased production of 11-deoxycorticosterone (DOC), a known hypertensinogenic mineralocorticoid[14]. This contention rests upon the results of both *in vitro* adrenal incubation studies and plasma steroid analyses[3]. In addition, other investigators have shown androgens capable of inhibiting 11β-hydroxylation *in vitro*[15] and we have found that the chronic treatment of rats with metyrapone, a non-steroidal competitive inhibitor of 11β-hydroxylation, also produces hypertensive disease[16].

It has been proposed that the mechanism(s) of this androgen-induced decrease in DOC metabolism involves both a reduction in the absolute levels of adrenal mitochondrial cytochrome P-450 and competition for binding to this cytochrome between androgen and DOC[6, 7]. This interaction of DOC with cytochrome P-450 is thought to represent an obligatory step in its conversion to corticosterone [9].

The results of the present studies are essentially consistent with and tend to solidify the aforementioned hypotheses. Of the three androgens administered (testosterone, DHA and androstenedione) only testosterone induced severe hypertensive disease. Similarly, only the adrenals from testosterone-treated rats showed a marked decrement in corticosterone production *in vitro*. Those androgens (DHA and androstenedione) which had little effect on adrenal 11β-hydroxylation did not produce significant alterations in blood pressure. The small increases in systolic blood pressure following DHA and androstenedione treatment might be attributable to a milder inhibition of DOC metabolism by those androgens. Such a possibility is suggested by the results of the adrenal homogenate incubations (Table 2) as well as *in vitro* studies[15, 17] in which these androgens were seen to inhibit 11β-hydroxylation but to a lesser extent than testosterone. In any event, in the present studies the degree of impairment in 11β-hydroxylation, as measured *in vitro*, corresponds very well with the blood pressure levels attained.

These studies also tend to clarify the probable mechanism of action of testosterone in the inhibition of 11β- and 18-hydroxylation. Whereas we have previously considered[6] both the reduced levels of cytochrome P-450 and decreased interaction (as reflected by the DOC-induced difference spectrum) of DOC with this cytochrome to be significant factors in the depression of DOC metabolism (11β- and 18-hydroxylation), it would now appear that the former may be of little consequence. All three androgens produced substantial and essentially equivalent reductions in the concentration of cytochrome P-450, yet only testosterone significantly affected adrenal 11β- and 18-hydroxylation. In addition, we have previously found[15] that during the course of testosterone administration, mitochondrial cytochrome P-450 levels are reduced prior to any demonstrable defect in 11β- and 18-hydroxylation. Similarly, other inhibitors of steroid

hydroxylases such as metyrapone seem to exert no effect on cytochrome P-450 levels[16, 18] *per se*.

On the other hand, the relative ability of DOC to interact with cytochrome P-450 does appear to parallel its rate of hydroxylation. Testosterone treatment resulted in comparable decreases in both the rates of corticosterone production *in vitro* and the magnitude of the DOC-induced spectral change. The administration of DHA or androstenedione, in contrast, affected neither of these parameters. When testosterone was added to adrenal mitochondria *in vitro* comparable inhibition of both the DOC-induced difference spectrum and the metabolism of DOC was again evident[6], an effect similar to that produced by metyrapone [18].

The observation[19] that testosterone induces a spectral change of its own, similar to that of DOC, is consistent with the thesis that both of these steroids interact with cytochrome P-450 in a similar manner. It is, therefore, quite probable that the increased secretion of DOC by the adrenal cortex of rats chronically treated with androgens is the result of a decreased capacity of this steroid to bind with cytochrome P-450, thereby preventing its conversion to corticosterone or 18-OH-DOC.

ACKNOWLEDGEMENTS

The authors are indebted to Mrs. Judith Colby and Mr. Luther Joseph for excellent technical assistance.

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