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Aldosterone Synthase Activity in the Y-1 Adrenal Cell Line

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The Y-1 adrenal cell line was shown to produce 20α-dihydroaldosterone from deoxycorticosterone. This compound was identified by GC–MS by comparison with the previously synthesized reference compound. Two other 18-hydroxylated metabolites were identified as 11β,18-dihydroxy-20α-dihydroprogesterone from endogenous cholesterol and 18-hydroxy-20α-dihydro-11-dehydrocorticosterone from DOC. The conditions necessary for the synthesis of these compounds are culturing in 20% serum-supplemented medium and repeated incubations with the substrate. The production of 11β-hydroxylated steroids and that of 18-oxygenated steroids is stimulated differently by ACTH and angiotensin II suggesting the expression of two different enzymes, cytochrome P-450$_{11\beta}$ and cytochrome P-450$_{aldo}$. The Y-1 cell line can secrete either 11β-hydroxylated steroids characteristic of the glucocorticoid pathway or 18-oxygenated steroids characteristic of the mineralocorticoid pathway, which *in vivo* are generally produced in two different zones of the adrenal cortex. This cell line should be an interesting model for the study of the molecular mechanisms regulating the expression of these two enzymes involved in the final steps of the steroidogenic pathways.

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INTRODUCTION

The final steps of hydroxylation in the synthesis of gluco- and mineralocorticoids are catalyzed by at least two distinct enzymes belonging to the cytochrome P-450$_{11\beta}$ family. Two similar enzymes, with different enzymatic activities, have been isolated from the human, rat and mouse adrenal cortex. Cytochrome P-450$_{11\beta}$, present in the fasciculata and glomerulosa, catalyzes the 11β-, 18-, and 19-hydroxylation of deoxycorticosterone (DOC) as well as the 11β- and 18-hydroxylation of 11-deoxycortisol. Cytochrome P-450$_{aldo}$, present only in the glomerulosa, catalyzes the 11β-hydroxylation of DOC and the 18-hydroxylation of corticosterone (B), followed by formation of aldosterone. In pathological conditions it can also catalyze the 11β-hydroxylation of 11-deoxycortisol and the 18-hydroxylation of cortisol, probably followed by the formation of 18-oxo-cortisol.

In the human cortex, two genes encoding for proteins with 93% homology have been described, CYP11B1 for 11β-hydroxylase and CYP11B2 for aldosterone synthase [1]. In the rat, two enzymes with molecular weights of 51.5 and 49.5 kDa and 81% homology have been characterized, corresponding to cytochrome P-450$_{11\beta}$ and cytochrome P-450$_{aldo}$, respectively [2, 3]. Four genes have been isolated from this species, CYP11B1 and CYP11B2 encoding the two proteins, and in addition CYP11B3 and CYP11B4; B3 has an unknown transcript, and B4 is a pseudogene [4]. In the mouse, two genes with 84% homology have been cloned and characterized, CYP11B1 encoding cytochrome P-450$_{11\beta}$ and CYP11B2 encoding cytochrome P-450$_{aldo}$ [5]. Two species, bovine and porcine, seem to be different from the others. Two enzymes with molecular weights of 48.5 and 49.5 kDa have been isolated from bovine adrenal mitochondria, but both are able to catalyze the 11β- and 18-hydroxylation of DOC and aldosterone synthesis [6]. Four different cytochrome P-450$_{11\beta}$ genes have been isolated and characterized in the bovine [7], and similar porcine enzymes have been discussed [8].

The Y-1 mouse adrenal cell line has been extensively used for the study of 11β-hydroxylase activity and its response to ACTH since it was established in 1966 [9]. In 1989, two newly discovered activities, 18- and 19-hydroxylase, were described with DOC as substrate [10, 11]. The major product of 18-hydroxylase activity is 18-hydroxy-20α-dihydrocorticosterone (18-OH-20α-DHB), a potential precursor of 20α-dihydroaldosterone (20α-DHaldo), as *in vivo* 18-hydroxycorticosterone is the precursor of aldosterone.

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In the present study the culture conditions necessary for the expression of cytochrome $P\text{-}450_{aldo}$ activity have been established and 20α-DHaldo identified. The effects of ACTH and AII on the 11β-hydroxylation and 18-oxygenation of DOC were also studied.

EXPERIMENTAL

All solvents were of analysis quality or HPLC grade from Merck and Carlo Erba. Steroids were purchased from Steraloids and Makor Chemicals. Commercially unavailable steroids were prepared by chemical and enzymatic methods and identified as the MO-TMS derivatives by GC–MS, as previously described [10–12].

The Y-1 mouse adrenal cell line (Flow Laboratories) was maintained in 5% serum-supplemented Ham's F10 medium (fetal calf serum and newborn calf serum; 1:1, v/v), as previously described [10]. Two days before the start of each experiment the cells were placed in 20% serum-supplemented medium with 5 nM ACTH or 10 μM angiotensin II (AII) for 2 pre-incubation periods of 24 h. Control cells received no hormone. Incubations were performed for 3 successive periods of 24, 48 and 72 h in 4 ml of fresh serum-supplemented medium, with or without ACTH or AII. The steroid precursor (50 μg) was added at the beginning of each incubation period in 0.01 ml of ethanol.

Steroids were extracted as previously described [13] and analyzed on HPLC with deoxycorticosterone-21-acetate as internal standard. Analysis was performed on a Varian 5000 with a 250 × 4 mm, 5 μm LiChrospher 100 RP-18 column (Merck) at 40°C, and a 30 × 4 mm, 25–40 μm LiChroprep RP-18 precolumn (Merck). A linear concentration gradient of acetonitrile/water from 15:85 to 75:25 (v/v) in 45 min at a 0.8 ml/min flow rate was used. Cellular proteins were quantified by the method of Lowry *et al* [14]. GC–MS analysis of the methoxime-trimethylsilyl (MO-TMS) derivatives of steroids was performed as previously described [12].

RESULTS

The metabolites produced from DOC by the Y-1 cell line in 10% serum-supplemented medium have been previously identified [10, 11]. In 20% serum-supplemented medium, several unknown metabolites appeared, which were identified as 18-oxygenated (Fig. 1). After purification on HPLC and analysis by GC–MS the most salient of the unknown metabolites was identified as the 18, 11β-hemiacetal form of 20α-DHaldo, and the small peak eluting just before it on HPLC its 18-aldehyde form. The GC characteristics and mass spectra of their MO-TMS derivatives are identical to those of the corresponding reference compounds prepared by chemical reduction of the 20-oxo group of aldosterone [12]. The mass spectrum of the MO-TMS derivative of the 18,11β-hemiacetal form is given in Fig. 2. The two major ions at m/z 504 (M-103) and 414 (M-103-90) result from the loss of 103 a.m.u. due to the cleavage of the TMS derivative of a primary alcohol. The fragment at m/z 205 represents the –CHOTMS–CH_2OTMS side chain. The ions at m/z 399 (M-90-118) and 386 (M-103-118) result from the cleavage of the 18,11β-hemiacetal ring.

Two other metabolites were tentatively identified by their GC characteristics and mass spectral data. The mass spectrum of the MO-TMS derivative of the first shows a molecular ion at m/z 607 and ions at m/z 504 (M-103) and 414 (M-103-90), showing the loss of a trimethyl ether of a primary alcohol (m/z 103). The base peak is observed at m/z 342 (M-265), this loss being specific for steroids with of an 18, 20, 21-trimethylsilyloxy structure [10]; the peak is thus tentatively identified as 18-hydroxy-20α-dihydro-11-dehydrocorticosterone. The other metabolite, as the MO-TMS derivative, has a molecular ion at m/z 593 corresponding to a completely derivatized oxopregnentriol and a base peak at m/z 117 corresponding to the loss of a side chain containing a

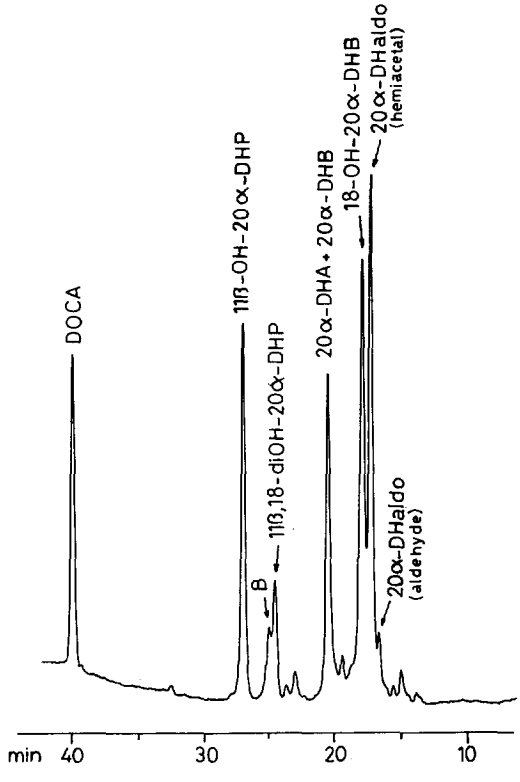

Fig. 1. HPLC analysis of the metabolites of DOC and the endogenous metabolites of cholesterol synthetized by the Y-1 cell line in 20% serum-supplemented medium (fetal calf serum/newborn calf serum, 1:1, v/v) after 72 h of incubation. Chromatographic conditions as in Experimental. Abbreviations as follow: DOCA, deoxycorticosterone-21-acetate; 11β-OH-20α-DHP, 11βhydroxy-20α-dihydroprogesterone; B, corticosterone; 11β, 18-diOH-20α-DHP, 11β,18-dihydroxy-20α-dihydroprogesterone; 20α-DHA, 20α-dihydro-11-dehydrocorticosterone; 20α-DHB, 20α-dihydrocorticosterone; 18-OH-20α-DHB, 18-hydroxy-20α-dihydrocorticosterone; 20α-DHaldo, 20α-dihydroaldosterone.

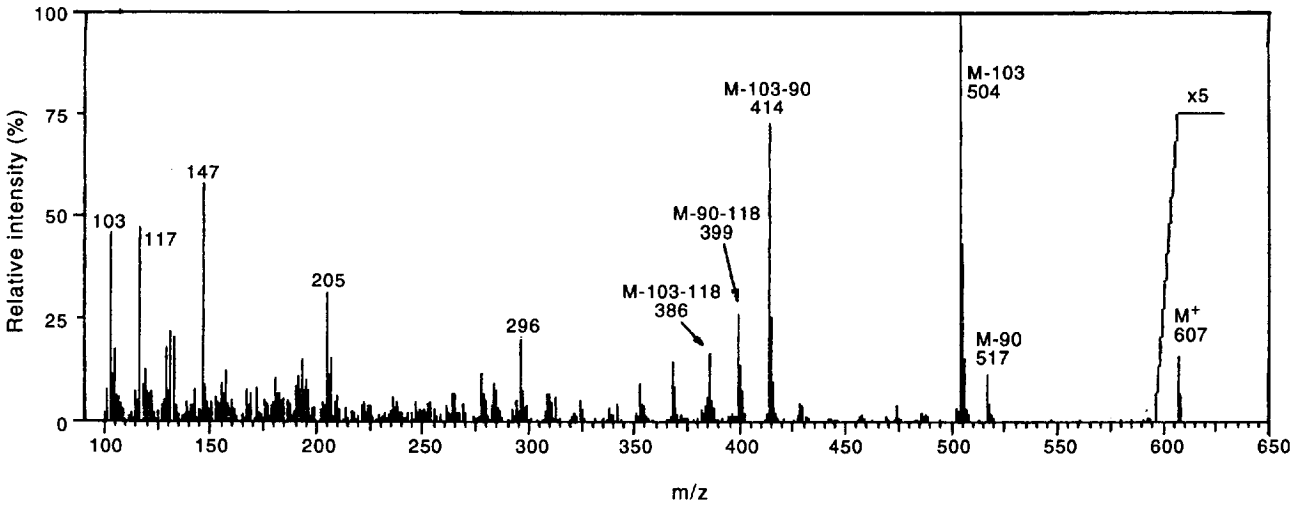

Fig. 2. Mass spectrum of the 18, 20α, 21-triTMS-3-MO derivative of the 18,11β-hemiacetal form of 20α-DHaldo produced by the Y-1 cell line from DOC.

20-trimethylsilyloxy-21-deoxy structure. Ions at 373 (M-117-103) and at 342 (M-117-103-31), as observed for 18-hydroxy-20α-dihydroprogesterone [15], are also observed. This compound is thus believed to be 11β,18-dihydroxy-20α-dihydroprogesterone (11β,18-diOH-20α-DHP), a metabolite of the most abundant product from the metabolism of cholesterol, 11β-hydroxy-20α-dihydroprogesterone (11β-OH-20α-DHP).

The effect of culture conditions, such as the concentration of serum in the media, repeated incubations of the substrate and the response to ACTH or AII, were investigated. The effect of several concentrations of AII on the formation of 20α-DHaldo and 18-OH-20α-DHB from DOC was studied. The maximum response to AII was obtained in cells treated with 10 μM (results not shown); this concentration was used for all further experiments.

An attempt to compare the effect of AII and ACTH on the formation of 11β-hydroxy, 18-hydroxy and 18-oxo metabolites in 1, 5, and 10% serum-supplemented medium was inconclusive, since 11β-hydroxylation was always complete and there was little if any 18-oxygenated metabolites in 1 and 5% serum-supplemented medium (results not shown). In 10% serum-supplemented medium, 18-hydroxylation was low and 20α-DHaldo was not detected. At this concentration of serum, 18-hydroxylation was similar in control and in ACTH treated cells, but doubled in AII treated cells. 20α-DHaldo was detected only in AII treated cells after 72 h of incubation.

Single incubation of substrate yielded little, if any, 20α-DHaldo, even after 72 h of incubation in cells cultured in 20% serum-supplemented medium. The maximum conversion to 20α-DHaldo in control cells (cells not treated with ACTH or AII) was 30.6% of the incubated DOC or 16.5 μg, after 72 h of incubation when the cells had been previously incubated for 24 and 48 h periods with the substrate (Table 1). In similar incubation conditions, 34.6% of the incubated B or 17.3 μg was metabolized to 20α-DHaldo (Table 2).

The comparison of the effects of ACTH and AII on the metabolism of DOC into 18-oxygenated steroids in 20% serum-supplemented medium is shown in Table 1. There was no significant effect of ACTH on the production of 18-OH-20α-DHB and 20α-DHaldo. Compared to the control, in cells treated with AII there

Table 1. Comparison of the effects of ACTH and angiotensin II (AII) on the biosynthesis of 18-hydroxy-20α-dihydrocorticosterone (18-OH-20α-DHB) and 20α-dihydroaldosterone (20α-DHaldo) from deoxycorticosterone (DOC) by Y-1 cells in 20% serum-supplemented medium

		Control	ACTH	AII
18-OH-20α-DHB*	24 h	2.0	1.4	2.6
		(16.0)	(11.4)	(22.6)
	48 h	4.8	4.5	6.1
		(38.4)	(37.8)	(53.7)
	72 h	5.7	5.2	5.3
		(44.9)	(43.3)	(46.9)
20α-DHaldo*	24 h	1.4	1.0	1.4
		(11.1)	(8.4)	(12.7)
	48 h	1.9	1.7	3.3
		(15.4)	(14.6)	(29.4)
	72 h	3.8	3.3	5.0
		(30.6)	(27.6)	(44.0)

*Metabolites expressed in μg/mg of protein and in percent of DOC incubated (values in parentheses). Values are the mean of duplicate flasks with two measurements per flask in a representative experiment. The range of variation between replicates is less than ± 4%.

Y-1 cells were incubated with 50 μg DOC (38 μM) for successive periods of 24, 48 and 72 h. ACTH and AII were added at the beginning of each incubation period at a concentration of 5 nM and 10 μM, respectively. Cells were pre-treated with the same concentration of ACTH or AII for two periods of 24 h before the beginning of the incubations. Control cells received no ACTH or AII.

Table 2. Comparison of the effects of ACTH and angiotensin II (AII) on the biosynthesis of 18-hydroxy-20α-dihydrocorticosterone (18-OH-20α-DHB) and 20α-dihydroaldosterone (20α-DHaldo) from corticosterone by Y-1 cells in 20% serum-supplemented medium

		Control	ACTH	AII
18-OH-20α-DHB*	24 h	0.5	0.4	0.8
		(4.8)	(3.5)	(7.2)
	48 h	2.3	0.5	3.8
		(21.3)	(3.8)	(34.5)
	72 h	3.6	3.0	3.6
		(33.0)	(24.9)	(32.9)
20α-DHaldo*	24 h	0.7	0.6	0.9
		(6.0)	(5.3)	(8.4)
	48 h	2.1	1.0	3.7
		(18.9)	(8.1)	(33.6)
	72 h	3.8	3.4	4.3
		(34.6)	(28.4)	(39.1)

*Metabolites expressed in μg/mg of protein and in percent of corticosterone incubated (values in parentheses). Values are the mean of duplicate flasks with two measurements per flask in a representative experiment. The range of variation between replicates is less than ± 4%.

Y-1 cells were incubated with 50 μg corticosterone (36 μM) for successive periods of 24, 48 and 72 h. ACTH and AII were added at the beginning of each incubation period at a concentration of 5 nM and 10 μM, respectively. Cells were pre-treated with the same concentration of ACTH or AII for 2 periods of 24 h before the beginning of the incubations. Control cells received no ACTH or AII.

was a significant increase in the production of 20α-DHaldo after 48 h of incubation (15.4–29.4%) and after 72 h of incubation (30.6–44.0%). If one considers both 18-oxygenated compounds, 18-OH-20α-DHB being a precursor of 20α-DHaldo, the stimulation of their production by AII is also evident; 83.1% compared to 53.8% in the control cells after 48 h of incubation, and 90.9% compared to 75.5% in the control cells after 72 h of incubation. At this high concentration of serum (20%), the effect of the two hormones on 11β-hydroxylation cannot be studied since it is complete under all circumstances.

The effect of ACTH and AII on the metabolism of B, immediate precursor of 18-hydroxycorticosterone and aldosterone, is shown in Table 2. There was no stimulation by ACTH of the conversion of B to 18-OH-20α-DHB and to 20α-DHaldo. However, there was an increase in the combined production of both 18-oxygenated compounds in cells treated with AII, for example 68.1% compared to 40.2% in control cells after 48 h of incubation.

DISCUSSION

In this study the Y-1 cell line was characterized in terms of the synthesis of the end products of the mineralocorticoid pathway and shown to produce 20α-DHaldo as well as 18-OH-20α-DHB from DOC and corticosterone. Increased conversion to these compounds was observed in particular conditions, i.e. repeated addition of substrate over 72 h of incubation in 20% serum-supplemented medium. Aldosterone synthase activity is thus stimulated by substrate, which may induce enzyme synthesis or protect the enzyme from catalytic inactivation. The intermediates in the synthesis of 20α-DHaldo are 20α-reduced compounds which are rapidly produced from the 20-keto precursors due to high 20α-reductase activity of Y-1 cells. The conversion of DOC to 18-oxygenated steroids is more marked than that of corticosterone; it is possible that 20α-DHB is not as good a substrate for cytochrome $P\text{-}450_{aldo}$ as is 20α-DHDOC, in agreement with the common observation that DOC is a better precursor for the production of aldosterone than is corticosterone.

It has previously been shown that Y-1 cells cultured in serum-supplemented medium have increased 11β-hydroxylation as a function of increased serum concentration and incubation time and that ACTH strongly increases this activity [16]. However, while there is increased 18-hydroxylation as a function of increased serum concentration and incubation time, there is also a low level of inhibition of this activity as a function of ACTH treatment [16]. The results reported here, with 20% serum-supplemented medium show similar 18-hydroxylation both in control cells and in cells treated with ACTH when DOC is used as substrate. When corticosterone is the substrate, there is a significant decrease in this activity in cells treated with ACTH. It is possible that when DOC is the substrate the stimulation of 11β-hydroxylation by ACTH to produce 11βhydroxylated precursors (corticosterone and 20α-dihydrocorticosterone), could mask an inhibitory effect of ACTH on 18-hydroxylation.

The increased production of 18-oxygenated metabolites from DOC and corticosterone in response to AII was obtained with μM concentrations of the hormone, indicating a very low sensitivity of the cells towards the hormone. It has previously been reported that the total number of AII receptors in Y-1 cells is seven times lower than in bovine adrenal cells [17], and that in addition the poor response of Y-1 cells to AII may reflect an alteration of the mechanism of activation of protein kinase C [18].

A cell line has been established from a human adrenocortical carcinoma, NCI-H295, which synthesizes aldosterone and thus expresses the enzymatic activity characteristic of the mineralocorticoid pathway [19]. Even though the basal aldosterone production is lower than that of the Y-1 cell line, this cell line is a valuable model for studying AII responses, since production of aldosterone is considerably increased by relatively small doses of AII [20].

Our results show a difference between the effects of ACTH and AII on the formation of 18-oxygenated metabolites in the Y-1 cell line and suggest the involvement of two enzymes. The existence of the two enzymes in the mouse, cytochrome $P\text{-}450_{aldo}$ exclusively

in the glomerulosa, and cytochrome $P\text{-}450_{11\beta}$ in the fasciculata and glomerulosa, has been reported [5]. The transcripts of the two genes have been found in the Y-1 cell line, with a 10-fold higher level of the cytochrome $P\text{-}450_{11\beta}$ mRNA. Treatment by ACTH caused a short term (4 h) increase of the mRNA of $P\text{-}450_{aldo}$ and a decrease thereafter to below control levels. The level of the $P\text{-}450_{11\beta}$ mRNA showed no increase until 9 h after ACTH treatment, which was then sustained for 24 h. These results suggest that ACTH induces the two genes by different mechanisms, and are in agreement with the results reported in this paper.

The Y-1 cell line, previously considered as a fasciculata cell line, could thus possess both functional enzymes implicated in glucocorticoid and mineralocorticoid pathways, which normally are located in different zones of the adrenal cortex *in vivo*. Only cytochrome $P\text{-}450_{11\beta}$ is functional under normal culture conditions, catalyzing the 11β- and 19-hydroxylation of the 20α-reduced metabolites of DOC and progesterone, and strongly stimulated by ACTH. Cytochrome $P\text{-}450_{aldo}$ needs special conditions to be expressed, i.e. 20% serum-supplemented medium and repeated incubation of substrate. It catalyzes the 18-hydroxylation of the 20α-reduced metabolites of 11β-hydroxyprogesterone and corticosterone, and the formation of 20α-DHaldosterone; it is not stimulated by ACTH. The molecular mechanisms regulating the expression of the two enzymes thus need to be studied by the techniques of molecular biology.

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