J. Steroid Biochem. Molec. Biol. Vol. 37, No. 4, pp. 599–604, 1990
Printed in Great Britain.

0960-0760/90 $3.00 + 0.00

SYNTHESIS OF 19-NOR-ALDOSTERONE, 18-HYDROXY-19-NOR-CORTICOSTERONE AND 18,19-DIHYDROXYCORTICOSTERONE IN THE HUMAN ALDOSTERONE-PRODUCING ADENOMA

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(Received 19 March 1990)

Summary—The recently synthesized 18-C-steroid derivative, 19-nor-aldosterone(19-nor-aldo) and 18-hydroxy-19-nor-corticosterone(18-OH-19-nor-corticosterone) possess mineralocorticoid and hypertensinogenic activity. They and an additional newly synthesized steroid, 18,19-dihydroxycorticosterone[18,19$(OH)_2$-corticosterone], may play a role in the etiology and pathogenesis of disorders thought to be caused by steroids with mineralocorticoid and hypertensinogenic properties. In this study we provide evidence that 19-nor-aldo, 18-OH-19-nor-corticosterone and 18,19$(OH)_2$-corticosterone are produced *in vitro* by aldosterone-producing adrenal adenomas and adenoma of Cushing's syndrome, "silent" adrenal adenomas and the adjacent adrenal tissue. Measurable amounts of these steroids were found in the incubation fluids of adrenal tissues using specific RIAs performed after a sequence of HPLC systems. The rates of production of the three steroids were high in the aldosterone-producing adrenal adenomas and in adrenal hyperplasia compared with in either Cushing's adenoma or "silent" adenoma.

INTRODUCTION

The 19-nor steroid derivatives often possess higher mineralocorticoid and hypertensinogenic activities than their 19-substituted mother compounds [1, 2]. In particular, extensive biochemical and clinical studies have been performed with 19-nor-deoxycorticosterone (19-nor-DOC), a steroid identified urine of the rats [3] and human [4] but not in the plasma [5]. Two possibilities have been considered to explain this phenomenon: (a) 19-nor-DOC is formed and acts upon the main target organ of the mineralocorticoid, i.e. the kidney; or (b) it is formed beyond the target structure; it is a metabolite and plays no role in physiologic or pathologic events. The biosynthetic pathway leading to 19-nor-DOC has been proposed as follows: DOC → 19-OH-DOC → 19-oxo-DOC → 19-COOH-DOC → 19-nor-DOC. The last step is believed to take place extra-adrenally, most likely in the kidney. Tetra-hydro-19-nor-DOC appeared only in the urine, not in other biological fluids [6]. Our present findings contradict the hypothesis that the 19-nor-corticosteroids are not elaborated by the adrenals, in that we detected 19-nor-aldosterone(19-nor-aldo) in the incubation medium of the adrenal adenoma as well as in the surrounding adrenal tissue. Additionally 18-hydroxy-19-nor-corticosterone(18-OH-19-nor-cortico-sterone) and 18,19-dihydroxycorticosterone[18,19$(OH)_2$-corticosterone] have been found in the incubates.

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EXPERIMENTAL

Patients

Adrenal tissue removed from 10 patients was evaluated. The diagnoses were: aldosterone-producing adenoma with hypertension ($n = 4$), adrenal hyperplasia ($n = 1$), Cushing's syndrome ($n = 1$) and "silent" adrenal adenoma ($n = 4$), two with hypertension and two without. Patients with aldosterone-producing adenomas

and hyperplasia presented with hypertension, hypokalemia, suppressed plasma renin activity and an increase in plasma and urinary aldosterone values. The patient with Cushing's syndrome had elevated plasma and urinary levels of cortisol accompanied by normal plasma renin activity and aldosterone concentrations. No hormonal abnormalities were detected in the patients with "silent" adenomas. Samples of adjacent non-tumorous tissue were obtained from three patients with aldosterone-producing adenomas and one each with Cushing's syndrome and "silent" adenoma.

Incubation of adrenal tissue

Immediately following removal of the adrenal tissue samples were placed in ice-cold Krebs–Ringer bicarbonate solution, pH 7.4, containing 0.2% glucose and bovine serum albumin (0.2%) (KRBSA). After subsequent washing with KRBSA, the tissue was minced and preincubated in 10 ml of fresh KRBSA at 37°C for 30 min in an atmosphere of 95% oxygen and 5% carbon dioxide. The medium was removed after centrifugation and replaced by 10 ml of fresh KRBSA. After incubation for 4 h the medium was centrifuged (30 min, 300 rpm). The supernatant was stored at −20°C until assay. An incubation period of 4 h is the maximal time for the *in vitro* production of aldosterone and 18-hydroxy-corticosterone as shown in preliminary experiments (data not shown).

Steroids and antibodies

Commercially available steroids were purchased from Sigma (Munich, F.R.G.). Uncommon steroids such as 18,19$(OH)_2$-corticosterone, 18-OH-19-nor-corticosterone and 19-nor-aldo were synthesized in the Department of Biotechnology of the Tel-Aviv University by one of the authors [7–9]. Antibodies were raised in white New Zealand rabbits following immunization with the respective steroid-3-oxime-bovine serum albumin conjugates. Aliquots of supernatants (5–10 ml), supplemented with the respective tritium-labelled steroids (3000 cpm) used for recovery monitoring, were extracted with Sep-pak C_{18} columns, dried under a stream of compressed air, redissolved in 0.3 ml of ethanol containing 0.1% triethylamine and chromatographed in a reverse-phase HPLC system.

An autosampler (Model 8480), pump (Model 8800), u.v. detector (Model 8480), all manufactured by Spectra Physics HPLC equipment (Darmstadt, F.R.G.), and, fraction collector (Model Frac-100, Pharmacia, Freiburg, F.R.G.) with a reversed phase column (Ultrasphere ODS, 5 μ, Beckman, Munich, F.R.G.) were used with two subsequent solvent systems. System I–water:acetonitril:methanol (72:23:5 by vol), flow rate 2 ml/min, R_t of 18,19$(OH)_2$-corticosterone, 18-OH-19-nor-corticosterone, 19-nor-aldo, 18-OH-corticosterone and Aldo were 16, 21, 28, 33 and 40 min, respectively. System II—water:tetrahydrofuran:methanol (78:17:5 by vol), flow rate 1.5 ml/min, R_t of 19-nor-aldo and 19-OH-aldo were 20 and 14 min, respectively. Aliquots of the respective fractions were evaporated under a stream of compressed air and the steroid content was measured using the specific RIA. HPLC fractions were reconstituted with a 0.2% solution of ethylene glycol water (100 μl). The respective tritium-labelled steroid (3000 cpm) and antibody (final dilution of antibodies: 18,19$(OH)_2$-corticosterone, 1:20000; 18-OH-19-nor-corticosterone, 1:3500; 19-nor-aldo, 1:1750 in volumes of 0.25 ml) were added and the mixtures were incubated (4°C, 24 h). Unbound radioactivity was then absorbed on the dextran-coated charcoal suspension and following centrifugation, the radioactivity of the antibody-bound steroid was measured in a liquid scintillation spectrometer (Wallac 1410, Pharmacia, Freiburg, F.R.G.). Aldosterone, 18-OH-corticosterone and 19-nor-aldo were determined according to methods published elsewhere [10–12]. All calculated values were corrected with individual recovery data.

HPLC–MS systems

We used a modified Finnigan MAT 4021 mass-spectrometer with a thermospray model (Finnigan MAT, Bremen, F.R.G.) connected to a Waters 600 MS multisolvent delivery system and a Waters ODS reverse phase HPLC column (15 cm × 3.9 mm, 5 μ) (Waters, Milfold, Mass) with a flow rate of 1.4 ml/min and a gradient system of methanol–water, 30–70%. The thermospray conditions were: vaporizer temperature, 122°C; aerosol temperature, 195°C. The MS conditions were: positive ionization selected ion monitoring (SIM, MID) in 1 s/scanning periods. Samples were dissolved in ethanol containing 0.1% triethylamine and injected in a HPLC.

Table 1. Cross-reactivity between the antibody (Ab) of 18,19-dihydroxycorticosterone, 18-hydroxy-19-nor-corticosterone and 19-nor-aldosterone and various steroid compounds.

	Ab:18,19(OH)$_2$-B	18-OH-19-nor-B	19-nor-aldo
	(% cross-reaction)		
18,19(OH)$_2$-B	100	0.1	<0.02
18-OH-19-nor-B	3.2	100	<0.02
18-OH-B	1.5	3.8	0.08
Aldo	<0.02	<0.02	193
19-nor-aldo	<0.02	0.5	100
18-OH-A	1.5	6.7	0.46
18-OH-DOC	0.6	1.2	19
Cortisol (F)	<0.02	<0.02	<0.02
Cortisone	<0.02	<0.02	<0.02
6-OH-F	<0.02	<0.02	<0.02
18-OH-progesterone	<0.02	<0.02	0.2
Corticosterone	<0.02	<0.02	0.29
19-OH-aldo	<0.02	<0.02	13
Tetrahydro-aldo	<0.02	<0.02	0.87
17-OH-progesterone	<0.02	<0.02	<0.02

A: dehydrocorticosterone; DOC: deoxycorticosterone; 18,19(OH)$_2$-B: 18,19-dihydroxycorticosterone; 18-OH-19-nor-B: 18-hydroxy-19-nor-corticosterone; 19-nor-aldo: 19-nor-aldosterone.

RESULTS

Evaluation of the assays

The sensitivity of the assay of 18,19(OH)$_2$-corticosterone, 18-OH-19-nor-corticosterone and 19-nor-aldo was 10, 5 and 10 pg, respectively. The cross-reactivity of their respective antibodies is shown in Table 1. Those steroids that would potentially interfere with the assay were eliminated by HPLC. The overall recovery was 50–60% for 18,19(OH)$_2$-corticosterone and 40–50% for 18-OH-19-nor-corticosterone and 19-nor-aldo. The interassay variation was 13.9% ($n = 15$) for 18,19(OH)$_2$-corticosterone; 15.0% ($n = 15$) for 18-OH-19-nor-corticosterone and 15.9% ($n = 15$) for 19-nor-aldo. The intraassay variation was 8.5% ($n = 6$), 9.7% ($n = 6$), and 11.2% ($n = 6$), respectively.

Identification studies

Samples were analyzed in a procedure similar to that used for the determination of 19-nor-aldo in the incubation media. After the last step of the isolation procedure, i.e. the second HPLC system, one aliquot was injected in the HPLC–MS system. The results are shown at the bottom of Fig. 1. The upper part shows the picture obtained after the injection of synthetic 19-nor-aldo. No similar experiment was done for 18-OH-19-nor-corticosterone and 18,19(OH)$_2$-corticosterone in the incubation fluids of adrenal tissue and adrenal tumor tissue. However, both steroids were identified in samples of human urine [16]. For these steroids identification was possible by the same retention time of the fractions isolated from the incubation fluids and standard steroids. Radioimmunological results with specific antibodies also supported their identity.

Experimental studies

The release of aldosterone and 18-OH-corticosterone from during adrenal incubation is demonstrated in Table 2. These mineralocorticoids were also present in the incubation media of the adenomas obtained from patients with Cushing's syndrome and "silent" adenomas.

Measurable 19-nor-aldo was present in all tissues except for one sample of adjacent

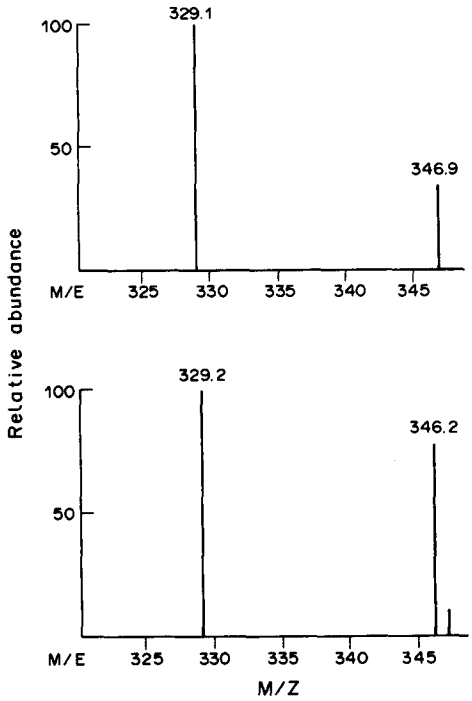

Fig. 1. Mass spectra of synthetic 19-nor-aldosterone (upper) and the sample of 19-nor-aldosterone isolated from the adrenal incubation medium (lower).

Table 2. Aldosterone and 18-hydroxycorticosterone values in the incubation medium of adrenal adenomas, hyperplastic tissue and adjacent adrenal tissues

Tissue	Aldosterone (pg/mg tissue)	18-OH-B (pg/mg tissue)
APA:		
No. 1	71	142
No. 2	80	210
No. 3	45	20*
No. 4	152	357
Adrenal hyperplastic tissue	105	341
Adenoma (Cushing's syndrome)	5	18
"Silent" adenoma with HT:		
No. 1	16	24
No. 2	15	15
"Silent" adenoma without HT:		
No. 1	6	19
No. 2	2	5
Adjacent adrenal tissue:		
No. 1	6	18
No. 2	8	29
No. 3	9	1.1
No. 4	16	41
No. 5	27	64

18-OH-B: 18-hydroxycorticosterone; APA: aldosterone-producing adenoma; HT: hypertension;
*Technical mistake is considered.

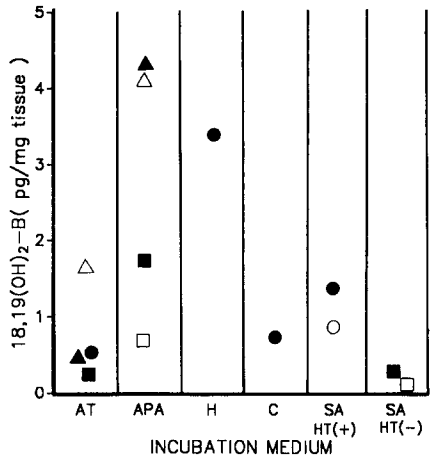

Fig. 3. 18,19-Dihydroxycorticosterone(18,19(OH)$_2$-B) in the incubation medium of adjacent adrenal tissue (AT) (■: No. 1, △: No. 3, ▲: No. 4, ●: No. 5 of AT), aldosterone-producing adenoma (APA) (■: No. 1, □: No. 2, ▲: No. 3, △: No. 4 of APA), adrenal hyperplastic tissue (H), adenoma of Cushing's syndrome (C), "silent" adenoma with hypertension [SA, HT (+)] [●: No. 1, ○: No. 2 of SA, HT (+)] and "silent" adenoma without hypertension [SA, HT (−)] [■: No. 1, □: No. 2 of SA, HT (−)].

adrenal tissue and one "silent" adenoma. The concentration of this hormone was especially high in samples from two aldosterone-producing adenomas and in the sample from a hyperplastic adrenal (Fig. 2). The 18,19(OH)$_2$-corticosterone was detected in all samples except for one specimen of adjacent adrenal tissue (Fig. 3). The concentration of 18,19(OH)$_2$-corticosterone in the incubation medium of the aldosterone-producing adenomas and of adrenal hyperplasia was high. Relatively high values were obtained in the "silent" adenoma with hypertension. 18-OH-19-nor-corticosterone was found in all samples. Production of 18-OH-19-nor-corticosterone was high in the aldosterone-producing adenomas and in adrenal hyperplastic tissue (Fig. 4).

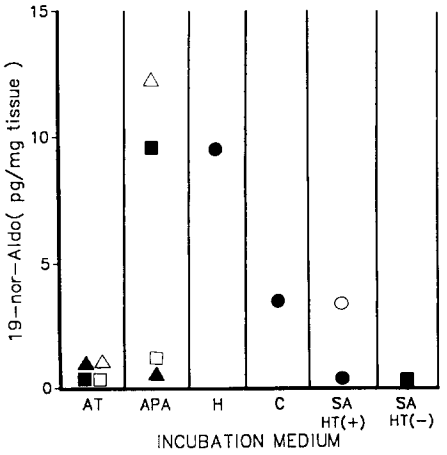

Fig. 2. 19-Nor-aldosterone(19-nor-aldo) in the incubation medium of adjacent adrenal tissue (AT) (■: No. 1, □: No. 2, △: No. 3, ▲: No. 4 of AT), aldosterone-producing adenoma (APA) (■: No. 1, □: No. 2, ▲: No. 3, △: No. 4 of APA), adrenal hyperplastic tissue (H), adenoma of Cushing's syndrome (C), "silent" adenoma with hypertension [SA, HT (+)] [●: No. 1, ○: No. 2 of SA, HT (+)] and "silent" adenoma without hypertension [SA, HT (−)] [■: No. 1 of SA, HT (−)].

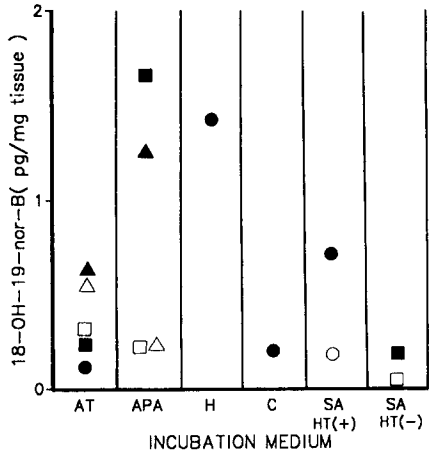

Fig. 4. 18-Hydroxy-19-nor-corticosterone(18-OH-19-nor-B) in the incubation medium of adjacent adrenal tissue (AT) (■: No. 1, □: No. 2, △: No. 3, ▲: No. 4, ●: No. 5 of AT), aldosterone-producing adenoma (APA) (■: No. 1, □: No. 2, ▲: No. 3, △: No. 4 of APA), adrenal hyperplastic tissue (H), adenoma of Cushing's syndrome (C), "silent" adenoma with hypertension [SA, HT (+)] [●: No. 1, ○: No. 2 of SA, HT (+)] and "silent" adenoma without hypertension [SA, HT (−)] [■: No. 1, □: No. 2 of SA, HT (−)].

DISCUSSION

It is known that 19-nor-steroid derivatives often possess marked mineralocorticoid and hypertensinogenic activity [1, 2, 13]. The 19-nor-DOC was found to be elevated in patients with low-renin essential hypertension, primary aldosteronism and Cushing's syndrome [14, 15]. 19-Nor-aldosterone has also been detected in human urine with high urinary values reported in patients with Conn's syndrome [12]. 19-Nor-aldosterone could be detected in the incubation medium of aldosterone-producing adenoma, the adrenal tissue adjacent to the tumor, hyperplastic adrenal tissue removed from a patient suffering from hypertension and hypokalemia, the adenoma of a patient with Cushing's syndrome and from patients with "silent" adenomas. The concentration of 19-nor-aldosterone was high in the incubation medium of two cases of aldosterone-producing adenoma and one hyperplastic adrenal. Shackleton *et al.* [6] have shown that tetrahydro-19-nor-DOC was present in the urine but not in other body fluids, leading to the assumption that 19-nor-DOC is formed in renal tissue from the presumed precursor steroid 19-oic-acid. The same author assumed that the other C-19 derivatives of 11-deoxy-corticosterone should also be elaborated extra-adrenally. The results presented here for 19-nor-aldosterone are somewhat contradictory to those of Shackleton *et al.*, at least concerning the formation of this steroid, in that it is produced, at least in part, by the adrenal tissue and by adrenal tumors.

The 18,19$(OH)_2$-corticosterone and 18-OH-19-nor-corticosterone compounds were recently synthesized [8, 9] and have been detected in human urine [16]. The hypertensinogenic properties of 18-OH-19-nor-corticosterone has also been reported [17]. We found high values of these steroids in the incubates of aldosterone-producing adenoma and also detected them in the samples of adjacent adrenal tissues. Both steroids were identified in human urine samples by mass spectrometric analysis [16]. Their identification in the incubation fluid of human adrenal tissues could not be confirmed until now. However, the retention times of these steroids were identical with those obtained by two different HPLC-systems with synthetic 18-OH-19-nor-corticosterone and 18,19$(OH)_2$-corticosterone. Their identity was also confirmed by radioimmunologic reaction with specific antibodies. 19-Nor-aldosterone was identified by mass spectrometry, both in samples of human urine [12] and in the incubation fluid of human adrenal adenoma, and in addition, by confirmative indices of HPLC and radioimmunologic data. The results are convincing concerning the identity of the three steroids produced by the adrenals as described in this study.

Acknowledgements—The authors thank Tom Iwuanyanwu and Ms Sandy Pelus for assisting in manuscript preparation.

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