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IDENTIFICATION OF ALDOSTERONE METABOLITES FORMED BY A6 CELLS

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Summary—The A6 cell line of the toad kidney is well known to form an Na^+ transporting tight epithelium in culture and is often used as an experimental model for Na^+ transport systems. Although it has been shown that A6 cells can convert aldosterone to polar metabolites, these metabolites have not been identified. Therefore, in this study, we tried to identify the metabolites of aldosterone formed by A6 cells in culture. A6 cells at confluence were incubated with serum-free culture media containing [^{3}H]aldosterone. When radioactive compounds in incubation media were separated by reversed phase high-pressure liquid chromatography (HPLC), four fractions (fractions A–D) were obtained. Fraction A, a mixture of two components, comprised the majority of metabolites formed. The more polar material (fraction A-1) and the less polar material (fraction A-2) of fraction A contained 47–71 and 9–19% of total radioactivity, respectively. When incubated in cell-free media, fraction A-2 was found to be unstable and partially converted to fraction A-1. Fraction B, 0.7–1.5% of total radioactivity, and fraction C, 8–21% of total radioactivity, cochromatographed with isoaldosterone and D-aldosterone, respectively. Fraction D, 4–8% of total radioactivity, was a mixture of two components, which cochromatographed with 3β,5β-tetrahydroaldosterone and 5α-dihydroaldosterone, respectively. In order to identify fraction A-2 material, large-scale cultures were performed and fraction A-2 was separated and purified by reversed phase HPLC. The purified material was analyzed by fast atom bombardment mass spectrometry and nuclear magnetic resonance spectroscopy. These two procedures unambiguously revealed that this material was 6β-hydroxyaldosterone. These results demonstrate that aldosterone can be converted to at least four metabolites by the incubation with A6 cells, and that major metabolites are polar compounds, a portion of which is 6β-hydroxyaldosterone.

INTRODUCTION

A6 cells, a continuous cell line derived from kidney of *Xenopus laevis*, are well known to form typical tight epithelia in culture when grown on permeable supports. Gnionsahe *et al.* [1] recently reported that the origin of this cell line might be the distal straight tubule of *Xenopus* nephron. Both high- and low-affinity aldosterone binding sites have been demonstrated in the nucleus [2, 3] and in the cytosol of A6 cells [3]. Moreover, these cells have a capacity to convert corticosterone to 6β-hydroxycorticosterone and these compounds are capable of stimulating active Na^+ transport in A6 cells [4]. Watlington *et al.* [2] also studied the metabolism of [^{3}H]aldosterone by A6 cells. Upon TLC of ethanol extracts of whole cells, a radioactive material that cochromatographed with aldosterone and a polar metabolite were found. However, this polar metabolite has not been identified.

Therefore, in the present study we have investigated the metabolism of aldosterone in A6 cells in order to define more completely the metabolites of aldosterone. We have found that aldosterone is converted to 6β-hydroxyaldosterone and to Ring-A-reduced metabolites (5α-dihydroaldosterone and 3β,5β-tetrahydroaldosterone) by A6 cells.

EXPERIMENTAL

Compounds

[1,2-^{3}H]Aldosterone (46 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, England. [^{3}H]Aldosterone was purified by reversed phase high-pressure liquid chromatography (HPLC) before use. D-Aldosterone, isoaldosterone, 5α-dihydroaldosterone, 3β,5β-tetrahydroaldosterone, 3α,5β-tetrahydroaldosterone, 3β,5α-tetrahydroaldosterone, plus β-

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glucuronidase and sulfatase preparations from Helix pomatia (type H-2), were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.

Cells in culture

Cells used in the present study were obtained from the American Type Collection at passage 69. Passages 70–80 were used for experiments. The cells were grown at 26°C in a humidified atmosphere of 1% CO_2 in air. The growth medium was Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine (2 mM) and glucose (1000 mg/l) diluted by 15% with distilled water to amphibian osmolarity. $NaHCO_3$ (8 mM), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum were added to this medium. Cells were seeded at a density of 2–4 $\times$ 10^4 cells/cm^2 in 10 cm tissue culture dishes and fed twice weekly. When they reached confluence and exhibited dome formation (10–14 days), they were used for experiments. The number of cells per culture dish was 1×10^7.

Experiment A

Cells grown in tissue culture dishes were incubated for 24 h with 5 ml serum-free DMEM containing 4 nM [^{3}H]aldosterone. As a control experiment, 4 nM [^{3}H]aldosterone was incubated for 24 h with a cell-free incubation medium.

Experiment B

Cells grown in tissue culture dishes were incubated for periods of time ranging from 1 to 24 h with 5 ml serum-free DMEM containing 2 nM [^{3}H]aldosterone. In another series of experiments, cells were incubated for 24 h with various concentrations (1–100 μM) of aldosterone and 2 nM [^{3}H]aldosterone.

Experiment C

Large-scale cultures (18 10-cm dishes) were carried out to obtain a large amount of the polar metabolite of aldosterone. Cells were incubated with 300–500 μg ($1.7–2.8 \times 10^{-4}$ M) aldosterone in serum-free medium for 24 h.

Preparation of the incubated samples for analysis

Extraction of aldosterone metabolites was carried out using Sep-pak C_{18} cartridges (Waters Associates Inc., Milford, Mass., U.S.A.) as described by Shackleton and Whitney [5].

The separation of aldosterone metabolites

Methanol eluates from Sep-pak C_{18} cartridge extraction were submitted to reversed phase HPLC using a pump system (model CCPE; TOSOH Co., Tokyo, Japan) and a TSK-gel ODS-80 Tm column (4.6 $\times$ 150 mm) or 7.8 $\times$ 300 mm; TOSOH Co., Tokyo, Japan). A column was operated at room temperature and the flow rate was 1 ml/min. The solvent systems used for analysis are shown in Table 1. By HPLC using solvent system 1, 3β,5β-tetrahydroaldosterone, 3α,5β-tetrahydroaldosterone and 3β,5α-tetrahydroaldosterone could be well separated; relative retention times for these compounds (with aldosterone as a reference) were 1.46, 1.59 and 1.83, respectively. However, the separation of 3β,5β-tetrahydroaldosterone from 5α-dihydroaldosterone could only be accomplished by HPLC in solvent system 5 and the retention times for these compounds were 11.7 and 16.9 min, respectively.

Enzymic hydrolysis

The sample was dissolved in 0.1 M acetate buffer at pH 4.6. Enzyme concentrations were 2460 Fishman unit/ml and 69 μM unit/ml with respect to β-glucuronidase and sulfatase activity, respectively. The solution was incubated for 48 h at 37°C. After the incubation, the liberated steroids were extracted with 3 vol of ethyl acetate.

Fast atom bombardment mass spectrometry (FABMS)

FABMS was performed on a mass spectrometer (JMS-DX303, JEOL, Tokyo, Japan). The FAB gun was operated with Xe gas. The accelerating voltage was 3 kV. The sample was analyzed in a liquid matrix (glycerol and triethanolamine).

Table 1. Conditions for reversed phase HPLC

Solvent system			Column size (mm)
1	Methanol	5	4.6 × 150
	Water	6	
2	Methanol	1	7.8 × 300
	Water	1	
3	Methanol	5	4.6 × 150
	Water	12	
4	Methanol	5	4.6 × 150
	Water	14	
5	Acetonitrile	1	4.6 × 150
	Water	3	

Nuclear magnetic resonance (NMR) spectroscopy

The NMR spectrum was recorded on NMR spectrometer (JMN-GX400, JEOL, Tokyo, Japan).

RESULTS

Experiment A

Cells were incubated for 24 h in media with 4 nM [^{3}H]aldosterone. After incubation the media contained 87–100% of incubated radioactivity. Incubation media were then applied to a Sep-pak C_{18} cartridge and 100% of the applied dose was recovered in methanol eluates. When the metabolites in methanol were separated by HPLC in solvent system 1, four fractions (fractions A–D) were present (Fig. 1, Table 2). Fraction A, which contained 70–86% of metabolites, was a mixture of two components which were separated by HPLC in solvent system 2. The more polar material (fraction A-1) comprised 47–71% of metabolites and had a retention time of 13.8 min. In solvents 3 and 4, retention times for this compound were 6.2 and 7.9 min, respectively. When an aliquot of this material was incubated with an enzyme preparation from *Helix pomatia*, little or no radioactivity became ethyl acetate extractable. The less polar material (fraction A-2), which contained 9–19% of metabolites, had retention times of 19.8 and 23.1 min in solvent systems 2 and 3, respectively. Fraction B, which contained 0.7–1.5% of total metabolites, cochromatographed with isoaldosterone in solvent system 1 (retention time 9.9 min). Fraction C comprised 8–21% of the recovered radioactivity and cochromatographed with D-aldosterone insolvent system 1 (retention time 13.5 min).

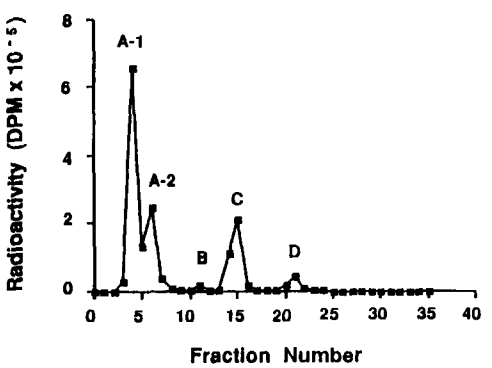

Fig. 1. HPLC of [^{3}H]aldosterone metabolites synthesized from A6 cells incubated with 4 nM aldosterone at 26°C for 24 h. The metabolites were separated on a TSK-gel ODS-80 Tm column (4.6 mm × 150 mm) by isocratic elution with methanol:water (5:6); flow rate 1 ml/min.

Table 2. [^{3}H]Aldosterone metabolites synthesized by A6 cells incubated with 4 nM aldosterone at 26°C for 24 h

	Expt 1 (%)	Expt 2 (%)	Expt 3 (%)
Fraction A	69.9	85.8	76.5
A-1	46.9	71.0	67.2
A-2	18.5	12.1	9.4
Fraction B	1.5	0.7	1.1
Fraction C	21.4	7.9	11.8
Fraction D	4.9	3.8	8.5
D-1	2.8	2.6	5.7
D-2	1.6	0.9	2.0

Fraction D, which contained 4–8% of total radioactivity and had a retention time of 19.6 min, was separated further by HPLC in solvent system 5 and two components were present. One of these compounds cochromatographed with 3β,5β-tetrahydroaldosterone (retention time 11.7 min) and the other with 5α-dihydroaldosterone (retention time 16.9 min). The ratio of 3β,5β-tetrahydroaldosterone to 5α-dihydroaldosterone in fraction D was 1.8:2.9.

The incubation of 4 nM [^{3}H]aldosterone with a cell-free incubation medium resulted in the formation of [^{3}H]isoaldosterone with a yield of 3.5%. When an aliquot of fraction A-2 was incubated for 24 h with a cell-free medium 33% of fraction A-2 was converted to fraction A-1.

Experiment B

Cells grown in tissue culture dishes were incubated for 1–24 h with 2 nM [^{3}H]aldosterone or for 24 h with 1–100 μM aldosterone and 2 nM [^{3}H]aldosterone. After incubation media were passed over a Sep-pak C_{18} cartridge, metabolites were separated by HPLC using solvent system 1. The synthesis of both fractions A and D increased linearly with time (Fig. 2). The synthesis of fractions A and D increased in proportion to the concentration of aldosterone in the medium (Fig. 3). However, the ratio of

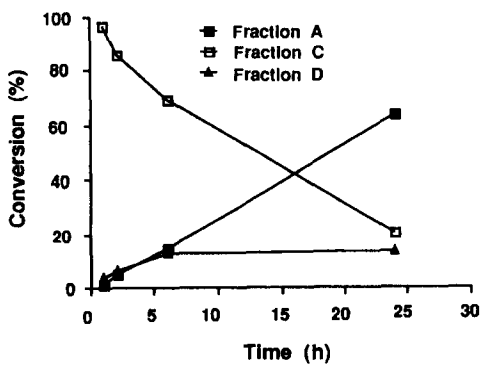

Fig. 2. Rates of synthesis of aldosterone metabolites by A6 cells incubated with 2 nM [^{3}H]aldosterone at 26°C.

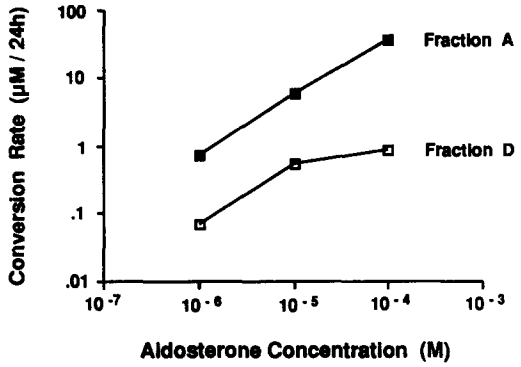

Fig. 3. Synthesis of aldosterone metabolites by A6 cells incubated with various concentrations of aldosterone for 24 h at 26°C.

fraction A-1 to fraction A-2 decreased in inverse proportion to the concentration of aldosterone (Table 3).

Experiment C

Cells were incubated for 24 h in media with aldosterone. After incubation media were passed over a Sep-pak C_{18} cartridge, metabolites were separated by HPLC. HPLC in solvent system 2 resulted in the separation of metabolites into fractions A-1 and A-2 and other metabolites. Fraction A-1 was further purified by HPLC in solvent system 3, 2 and 4. Fraction A-2 was purified by HPLC using solvent system 3. Fraction A-2 showed the positive reaction for blue tetrazolium (BT) and u.v. absorbance at 254 nm, whereas fraction A-1 exhibited a negative reaction for BT and no u.v. absorbance at 254 nm. Analysis of fraction A-1 by FABMS did not yield quasi-molecular ions, which were related to hydroxylated or reduced derivatives of aldosterone or to conjugated metabolites of these derivatives. Fraction A-2 was also analyzed by FABMS. In the positive ion spectrum of fraction A-2, ions were seen at mass-to-charge ratios of 377 and 399, which corresponded to $[M+H]^+$ and $[M+Na]^+$ of

Table 3. A6 cells were incubated with various concentrations of aldosterone in serum-free media for 24 h at 26°C

Aldosterone concentration (M)	Fraction A-1 (%)	Fraction A-2 (%)	Ratio A-1/A-2
10^{-6}	47.2	24.9	1.90
10^{-5}	30.1	28.9	1.04
10^{-4}	5.6	31.5	0.18

Fraction A-1 and A-2 in incubation media were separated by reversed phase HPLC.

monohydroxyaldosterone, respectively. In the negative ion spectrum of fraction A-2, an ion was observed at a mass to charge ratio of 375, which corresponded to $[M-H]^-$ of monohydroxyaldosterone. For NMR analysis, fraction A-2 was separated from the other metabolites by HPLC in solvent system 2. Fraction A-2 was purified twice by HPLC using the same solvent system. The purified material and D-aldosterone were submitted to ^{1}H-NMR spectroscopy and the spectra shown in Table 4 were obtained.

DISCUSSION

The present studies have shown that A6 cells have the ability to metabolize aldosterone effectively. The metabolites formed could be completely separated by HPLC and at least five metabolites were detected.

The most polar substance (fraction A-1) was a major metabolite of aldosterone. Though the elution pattern of fraction A-1 from an HPLC column indicated that this compound might be a steroid conjugate, this material could not be hydrolyzed with an enzyme preparation from *Helix pomatia*. Moreover, FABMS analysis of fraction A-1 revealed that this compound was neither a hydroxylated nor a reduced derivative of aldosterone, nor a conjugate of these derivatives.

Fraction A-2 was more polar than aldosterone and contained 9–19% of total radioactivity. FABMS analysis of this fraction indicated that

Table 4. The sample was dissolved in $CDCL_3$ and proton-NMR was recorded on an NMR spectrometer (JMN-GX400, JEOL)

Proton-NMR spectrum (aldosterone)			Proton-NMR spectrum (fraction A-2)		
δ(ppm)	Character	Assignment	δ(ppm)	Character	Assignment
1.260	s	19-H_3	1.452	s	19-H_3
1.309	s	19-H_3	1.497	s	19-H_3
2.813		17-H	2.824		17-H
3.464 (J = 11.5, 8.8)	dd	21-H_2	3.470 (J = 11.4, 8.9)	dd	21-H_2
3.616 (J = 11.5, 5.4)	dd	21-H_2	3.616 (J = 11.4, 5.3)	dd	21-H_2
4.245 (J = 18.1, 4.5)	dd	21-H_2	4.257 (J = 18.1, 4.5)	dd	21-H_2
4.393 (J = 18.1, 4.8)	dd	21-H_2	4.397 (J = 18.1, 4.4)	dd	21-H_2
4.597 (J = 6.3)	d	11-H	4.592 (J = 6.1)	d	11-H
4.834 (J = 5.9)	d	11-H	4.822 (J = 5.6)	d	11-H
5.035 (J = 5.1)	d	18-H	5.088 (J = 4.9)	d	18-H
5.425	s	18-H	5.483	s	18-H
5.725	s	4-H	5.826	s	4-H

fraction A-2 represented monohydroxyaldosterone. ^{1}H-NMR spectroscopic analysis of fraction A-2 revealed that this material was a mixture of two components and these two components were considered isomers judged from the result of the mass spectrometric analysis. In the ^{1}H-NMR spectrum of fraction A-2, protons at C-19 were observed at δ1.497 and 1.452. In contrast, signals of protons at C-19 in aldosterone appeared at δ1.309 and 1.260 (i.e. at a high magnetic field). This finding supported the assumption that fraction A-2 had a hydroxyl at C-6β [6–9]. In addition, the C-4 proton resonance shifted downfield by 0.10. This phenomenon also suggested that fraction A-2 possessed a hydroxyl at C-6β [10, 11]. Moreover, a peak corresponding to the proton at C-4 in the fraction A-2 molecule was sharp compared with that in aldosterone, indicating that the proton at C-6β was substituted in fraction A-2 [11].

It is noted that Kirk *et al.* [12, 13] have shown that 6β-hydroxyaldosterone exists in solution as two isomers, namely the 20-oxo and 18,20 hemiacetal forms. Two components found in fraction A-2 probably correspond to these two isomers. 6β-Hydroxyisoaldosterone and 6β-hydroxyaldosterone have been identified as polar metabolites formed from aldosterone by the incubation with a male rat liver microsomal fraction [13]. The possibility should be taken into consideration that fraction A-2 represents 6β-hydroxyisoaldosterone. While the signal for the 17-H of 6β-hydroxyisoaldosterone shifted downfield by >0.37 ppm as compared with aldosterone [12], the chemical shift for the 17-H of fraction A-2 is essentially identical to that of aldosterone. In addition, 6β-hydroxyisoaldosterone exists only in the 20-oxo form [12,13]. From these findings, it seems unlikely that fraction A-2 represents 6β-hydroxyisoaldosterone. Thus, identification of fraction A-2 as 6β-hydroxyaldosterone has been well established. As shown in experiment B, incubation of A6 cells with 10^{-4} M aldosterone resulted in the formation of larger amount of fraction A-2 as compared with fraction A-1. From these results, it is concluded that 6β-hydroxyaldosterone is an unstable compound and the fraction A-1 is a degradation product from the former substance. It is noted that isoaldosterone, a minor metabolite found in incubation media, is not a biosynthetic product, indicated by the result of experiment A.

The metabolism of [^{3}H]aldosterone by A6 cells was studied by Watlington *et al.* [2]. TLC of ethanol extracts of whole cells was performed. Radioactive material that cochromatographed with aldosterone and a polar derivative or metabolite(s) were found. This polar derivative probably corresponds to fraction A in our studies.

Monohydroxyaldosterone has been also found in rabbit urine as a metabolite of aldosterone [14]. The retention time for this compound in HPLC using solvent system 2 was close to that for fraction A-2. Therefore, it is likely that this compound represents 6β-hydroxyaldosterone.

Hydroxylated and Ring-A-reduced metabolites of aldosterone were also synthesized by rat kidney slices [15]. In addition, [^{3}H]aldosterone was transformed into several metabolites by subcellular fractions of rat kidney [16]. Nuclei and plasma membranes converted aldosterone to 5α-dihydroaldosterone and 3α,5α-tetrahydroaldosterone, whereas kidney cytosol metabolized aldosterone mainly to 5β-dihydroaldosterone and 3α,5β-tetrahydroaldosterone. It has been also found that 5α-reduced metabolites were synthesized when [^{3}H]aldosterone was incubated with toad urinary bladder [16].

The physiologic activities of aldosterone metabolites have been studied by several investigators. Reduced derivatives of aldosterone were shown to have mineralocorticoid activity when assayed in adrenalectomized rats or toad urinary bladders [17]. Interestingly, 19-hydroxyaldosterone was demonstrated to possess mineralocorticoid activity when measured in adrenalectomized rats or toad urinary bladders [18]. In addition, Grogan *et al.* [4] reported that 6β-hydroxycorticosterone could stimulate active Na^+ transport in A6 cells. Therefore, the possibility exists that 6β-hydroxyaldosterone also has mineralocorticoid activity and remains to be studied.

Addendum

During the editorial process for this manuscript another paper was published: "Synthesis of 6β-hydroxyaldosterone by A6 (toad kidney) cells in culture" by D. J. Morris *et al.* *Steroids* **55** (1990) 482–487.

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