Synthesis of 6β-hydroxyaldosterone by A6 (toad kidney) cells in culture

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Incubation of aldosterone with confluent layers of A6 (toad kidney) cells leads to its hydroxylation at the 6β-position. 6β-Hydroxyaldosterone is the major metabolite when the incubation is carried out at pH 6.8, whereas the product comprises 6β-hydroxy-17-isoaldosterone accompanied by some 6β-hydroxyapoaldosterone at pH 7.4. All products were identified by high-field ^{1}H nuclear magnetic resonance spectroscopy. Control experiments indicated that the side-chain isomerization to form the 17-iso and apo derivatives occurs after *the cytochrome P 450-dependent synthesis of 6β-hydroxyaldosterone.* (*Steroids* **55**:482–487, 1990)

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Introduction

We have recently identified, using high-field nuclear magnetic resonance (NMR) studies, the presence of 6β-hydroxyaldosterone **1** and 6β-hydroxy-17-isoaldosterone **2** among the major polar neutral metabolites synthesized when aldosterone is incubated with the microsomal fraction obtained from male rat liver.[1,2] The 6β-hydroxylation of other steroids by the liver of male rats has previously been reported,[3,4] and 6β-hydroxylation does occur to a small extent in the human metabolism of cortisol.[5–7] The presence of 6β-steroid hydroxylase has also been demonstrated in the A6 cell line derived from toad kidney, in which 6β-hydroxycorticosterone has been isolated when confluent layers of these cells are incubated with concentrations of corticosterone that produce active Na^+ transport.[8] 6β-Hydroxycorticosterone has recently been identified in the urine of male rats.[9]

The present studies were undertaken to determine whether aldosterone can also serve as a substrate for the 6β-steroid hydroxylase present in the A6 cell line and, hence, be a biologic source of this cytochrome P 450-dependent hydroxylated product.

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Experimental

Chemicals

[1,2-^{3}H]Aldosterone with a specific activity of 46.2 Ci/mmol was obtained from New England Nuclear Corporation (Boston, MA, USA). The [1,2-^{3}H]aldosterone was purified by high-performance liquid chromatography (HPLC) before use. Nonradioactive aldosterone was obtained from Andard-Mount (London) Ltd. (Wembley, Middlesex, UK).

In vitro incubation of aldosterone with A6 cells

Cells in culture. A6 cells (a continuous cell line derived from the kidney of *Xenopus paeris*) were obtained from the American Type Culture Collection at passage 69. Passages 71 through 78 were used for experiments. The cells were grown at 28 C in a humidified atmosphere of 1.7% CO_2 in air. The growth medium was Dulbecco's modified Eagle's medium (DMEM) with glutamine, glucose (1,000 mg/L), and pyruvate (catalog no. 430-

1600, GIBCO, Grand Island, NY, USA) and was diluted 15% with distilled water to adjust to amphibian osmolarity. To this medium were added $NaHCO_3$ (8 mM, pH = 7.4), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (GIBCO). Cells were seeded at a density of 2.5 to 5 × 10^4 cells/cm^2 in 10-cm tissue culture dishes and fed twice weekly. When they reached confluence and exhibited dome formation (10 to 14 days), they were used for experiments or were subcultured after removal from dishes with 0.25% trypsin plus 1 mM EGTA in calcium- and magnesium-free amphibian Ringer solution.

Twenty 10-cm tissue culture dishes (containing 5 to 10 × 10^6 cells/dish; approximately 5 mg of cell protein) from the same seeding were incubated with 5 ml of serum-free DMEM (pH 7.4) in each dish containing 1 × 10^{-4} M aldosterone and 0.176 μCi [^{3}H]aldosterone to prepare metabolites. One dish contained DMEM without cells as control. The medium was aspirated from the dishes at the end of 24 hours. Samples from the dishes containing cells were pooled and all medium was immediately frozen at −20 C.

A second set of incubation experiments was performed using the same conditions except that the DMEM was adjusted with 2.1 mM $NaHCO_3$ to pH 6.8.

Large-scale synthesis of polar aldosterone metabolites eluting between 36 and 42 minutes

The A6 cells were thawed at 4 C and extracted with 9 vol of acetone/ethanol (1 : 1 v/v) for 18 hours at 37 C. Following centrifugation at 600 × *g*, the cell extracts were evaporated to dryness under nitrogen and dissolved in 15% aqueous methanol. Aldosterone and its metabolites present in the incubation medium were extracted with Sep-pak C_{18} cartridges (Waters Associates, Milford, MA, USA) according to the method of Morris and Tsai.[10] Cartridges were first prepared by washing with MeOH (5 ml) and then with H_2O (5 ml). The incubation medium was extracted by passing through the Sep-pak cartridge, followed by washing with H_2O (5 ml) and elution with MeOH (5 ml). The MeOH extract was evaporated to dryness under N_2 and dissolved in 15% aqueous MeOH.

The combined cell and medium extracts were purified on a C_8 Zorbax (Dupont) column and metabolites of aldosterone were separated using a stepwise gradient of aqueous methanol (15% MeOH for 20 minutes, 35% MeOH for 30 minutes, and 50% for 35 minutes) at a flow rate of 1 ml/min at room temperature. The eluate was collected as 1-ml fractions in siliconized vials using a fraction collector. A total of eight to 10 injections were made to separate all of the incubation extract. An aliquot from each vial was mixed with 5 ml Instagel liquid scintillation fluid (Packard Instruments, Downers Grove, IL, USA) and counted for ^{3}H radioactivity.

High-pressure liquid chromatography

Metabolites eluting in the 36 to 40-minute fractions were collected and processed for the NMR studies reported here (see below). Other smaller metabolite fractions eluting between 31 and 33 minutes and between 33 and 35 minutes were saved for further structural determinations. For further purification, the combined 36- to 40-minute fractions were rechromatographed on a C_8 Zorbax column using Solvent System 7 (15% MeOH for 60 minutes, 25% MeOH for 30 minutes, followed by a linear gradient from 25% to 100% MeOH over 20 minutes) at 44 C.

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectra were determined at 400 MHz on a Bruker WH-400 spectrometer (University of London Intercollegiate Research Service, Queen Mary College), or at 500 MHz on a Bruker AM-500 spectrometer (National Institute for Medical Research, Mill Hill, London, UK). The solvent was methanol-d_4 ("ultra puriss" grade, >99.95 atom% ^{2}H) from Ciba-Geigy.

Two-dimensional ^{1}H homonuclear shift-correlated spectra (COSY-45)[11,12] were obtained with 2,048 data points in the f_2 dimension and zero-filling to 1,024 data points in the f_1 dimension (512 or fewer actual experiments were usually acquired) to achieve a symmetric data matrix on transformation. A shifted sine-bell window function was used.

Thermospray and plasmaspray high-performance liquid chromatography/mass spectrometry

Samples were analyzed on a VG-30-250 mass spectrometer (VG Maslab, Altrincham, Cheshire, UK) interfaced with a Waters 600 pump and controller (Waters Associates, Milford, MA, USA). The interface was a VG combined thermospray/plasmaspray with operating conditions optimized by repetitive analysis of cortisol.[13] Typical source and capillary temperatures were 245 C and 237 C, respectively. Isocratic separation was carried out on a 15-cm C_{18} column with either methanol : water, 50 : 50 (v/v) (plasmaspray), or methanol : aqueous 0.1 M ammonium acetate, 50 : 50 (v/v) (thermospray), as solvent system. The steroid of present interest gave a single peak under these conditions with a retention time of 4.0 minutes.

Plasmaspray ionization relies on the application of a discharge current to the plasmaspray electrode (typically, 275 μA) since no ammonium acetate is present in the solvent system to encourage ionization.

Gas chromatography/mass spectrometry

Samples were silylated by the addition of 50 μl BSTFA followed by heating at 60 C for 15 minutes. Gas chromatography/mass spectrometry (GC/MS) was carried out on a Hewlett-Packard 5970 MSD equipped with a 15-m SPD-1 fused-silica capillary column (Supelco). The sample was injected "splitless" at 50 C and, after 3 minutes, the temperature was rapidly taken to the "starting temperature" of 210 C. Thereafter, the temperature was programmed to 320 C and 3 C/min. The compound of interest had a retention time of 28.7 min-