The Possible Biological Role of Aldosterone Metabolites

DAVID J. MORRIS, PH.D., CHRISTOPHER J. KENYON, PH.D., SYED A. LATIF, PH.D., MARTIN MCDERMOTT, M.S., AND THEODORE L. GOODFRIEND, M.D.

SUMMARY Following the incubation of aldosterone with microsomes from liver of adrenalectomized male rates, a previously unidentified polar neutral metabolite of aldosterone, designated “peak A” material, was isolated and purified using high pressure liquid chromatography. This peak A material, which contains a reduced hydroxylated metabolite of aldosterone, was shown to possess 2% to 3.5% of the mineralocorticoid activity relative to aldosterone. When bovine adrenal glomerulosa cells were incubated with peak A material (3 and 10 µg/ml), the binding of 125I-angiotensin II was inhibited by 20% and 36%, respectively. The mineralocorticoid activity of the six possible ring A reduced metabolites was tested. The 5α-reduced metabolites were more potent than the 5β-, and the 3α- were more potent than the 3β-reduced metabolites. The renal and extrarenal transformations of aldosterone to the polar and nonpolar (reduced) metabolites and their possible role in the accepted mechanism of action of aldosterone is discussed. (Hypertension 5 (suppl I): I-35-I-40, 1983)

KEY WORDS • aldosterone • metabolites • physiological significance • kidney • sodium • potassium • renin

SIGNIFICANT quantities of several polar neutral metabolites and reduced metabolites of aldosterone are present in rat plasma and kidney during the hormone’s latent period before its physiological actions are observed. Similar polar neutral metabolites and reduced metabolites of aldosterone are also present in the liver. Studies in our laboratory have suggested that the metabolism of aldosterone is of importance in the mechanism of action of this hormone in the kidney, since the quantities of polar and reduced metabolites in the kidney correlate well with the magnitude of the physiological response. and these metabolites are present in significantly smaller quantities in the kidney of rats treated with the mineralocorticoid antagonist, spironolactone. These metabolites are also present in the nuclei of kidney cells isolated from rats treated with physiological quantities of 3H-aldosterone.

Recent experiments have shown that aldosterone is transformed in rat kidney slices to four of the six possible ring A-reduced metabolites of aldosterone and several polar neutral metabolites, and that significant portions of these transformations occur in the nucleus and plasma membrane of kidney cells. Several of the ring A-reduced metabolites of aldosterone have previously been shown to possess mineralocorticoid activity. To better understand their role in Na+ and K+ homeostasis and the pathogenesis of hypertension, the biological properties of several of these metabolites were investigated in the current studies.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River Laboratories) were bilaterally adrenalectomized under ether anesthesia at 6 weeks of age. Animals were then fed Purina Laboratory Chow and maintained on 0.9% NaCl as drinking water in a temperature- and light-controlled room. They were used for experiments 4–8 days after adrenalectomy.

Chemicals

1, 2-(3H)-aldosterone (46.2 µCi/mmol) and 4-14C-aldosterone (57.0 mCi/mmol) were obtained from New England Nuclear Corporation (Boston, Massachusetts). Nonradioactive aldosterone was obtained from Steraloids, Inc. (Witton, New Hampshire). 3β, 5β-tetrahydroaldosterone (THA) was obtained from Makor Chemicals (Jerusalem, Israel), 5α-dihydroaldosterone (DHA) and 5β-DHA were provided by Prof. D.N. Kirk, MRC, London, and 3β, 5α-THA was a generous gift of Professor M. Harnick, University of Tel Aviv, Israel. 3α, 5β-THA was prepared by incubating aldosterone with cytosol from male rat liver by the method of Ulick and Ramirez, and 3α, 5α-THA was prepared by incubating aldosterone with microsomes from female rat liver using a method modified from Kelly et al. The purity of labeled and
unlabelled aldosterone and of the reduced metabolites of aldosterone was verified chromatographically before use. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and NADPH, were obtained from Sigma Chemical Company.

**In Vitro Synthesis of Polar Aldosterone Metabolite (Peak A)**

The microsomal fraction from male adrenalectomized rat liver was isolated as previously described and incubated at a concentration of 10 mg protein/ml in the presence of 4-14C-aldosterone (50 μM; specific activity 22,200 DPM/nmol), 2% ethanol, 50 mM, Tris-HCl buffer, pH 7.4, 500 μM NADPH, 50 mM glucose-6-phosphate, 25 units per ml of glucose-6-phosphate dehydrogenase and 5 mM MgCl2 in a total volume of 1.0 ml. Large scale synthesis of peak A was accomplished by incubating aldosterone (10 mg) with large volumes of this incubation mixture (500 ml). The incubation mixture was constantly shaken at 37°C for 20 minutes in an atmosphere of air. The enzyme reaction was started by adding the microsomal fraction, and terminated by rapidly passing it through Sep-Pak C18-cartridges (Waters Associates) as described below.

**Extraction and Chromatographic Isolation of Aldosterone and Its Metabolites**

Aldosterone and its metabolites present in urine samples as well as those synthesized in the in vitro experiments were extracted using Sep-Pak C18 cartridges. The methanolic extract from the Sep-Pak was evaporated to dryness under nitrogen, redissolved in 72% aqueous ethanol, and separated on Sephadex DEAP-LH-20 columns (6 x 90 mm) into fractions containing neutral, acidic, glucuronide, sulfate, and disulfate steroid metabolites, as previously described. The first fraction (20 ml) from Sephadex DEAP-LH-20 chromatography, consisting of 72% aqueous ethanol (containing aldosterone and its neutral metabolites), was dried under nitrogen then redissolved in 15% aqueous methanol. Aldosterone and its metabolites were separated by HPLC using a DuPont Zorbax C18 reverse phase column and stepwise elution with 15% aqueous methanol (20 ml), 33% aqueous methanol (30 ml), and 50% aqueous methanol (30 ml) at a constant flow rate of 1 ml/min. The eluent was collected at 1.0-minute intervals, and aliquots counted directly in 5 ml Instagel scintillation fluid (Packard Instruments).

**Mineralocorticoid Bioassay in Adrenalectomized Rats**

Food was removed from adrenalectomized rats 16 hours before experimentation. On the morning of the tests, rats were made to urinate with a whiff of ether, then injected subcutaneously (s.c.) with 3 ml 0.9% NaCl and with various doses of aldosterone and aldosterone metabolites dissolved in 0.2 ml of a mixture of 0.154 M NaCl: ethanol (8:2, vol/vol). Control rats received vehicle alone. Rats were again induced to urinate with ether at 1 and 3 hours postinjection. Urine collection at 3 hours was analyzed for Na+ and K+ by flame photometry and for creatinine using a Beckman creatinine analyzer. Six rats were used for each dose of steroid. In all experiments, no significant differences in the quantities of creatinine excreted in the urine collected at hourly intervals were observed. This suggests that administration of aldosterone and its metabolites had no effect on the excretion of creatinine.

**Angiotensin II Binding Studies**

For studies of angiotensin receptors, suspensions of bovine adrenal glomerulosa cells were prepared by collagenase digestion, and angiotensin II was iodinated with 125Iodine by methods described previously. Eluates from HPLC columns were reconstituted in methanol. Conical polyethylene incubation tubes contained 150,000 cells, the aldosterone derivative or control, methanol at a final concentration of 1%, and 125I-angiotensin II (0.03 μCi) at a final concentration of 10-10 M in a volume of 0.5 ml. The buffer contained 115 mM NaCl; 4.6 mM KCl; 3.8 mM NaH2PO4; 16.2 mM Na2HPO4; and 11 mM glucose, at pH 7.4. Albumin was omitted. Incubations were performed at 37°C for 30 minutes, and were terminated by centrifugation. Inhibition of binding was calculated after subtraction of nonsaturable binding, which was determined by adding unlabelled angiotensin (10-9 M) to some tubes.

**Results**

**Large-Scale Synthesis of Peak A Material**

Following the large-scale incubation of aldosterone with microsomes from male rat liver, the mixture of aldosterone metabolites synthesized was separated by high pressure liquid chromatography (HPLC) into six major peaks of polar neutral metabolites, designated peaks A through F, as well as several peaks of reduced metabolites. Each individual peak of polar metabolites was separately and then purified by chromatography, consisting of 72% aqueous ethanol (containing aldosterone and its neutral metabolites), was dried under nitrogen then redissolved in 15% aqueous methanol. Aldosterone and its metabolites were separated by HPLC using a DuPont Zorbax C18 reverse phase column and stepwise elution with 15% aqueous methanol (20 ml), 33% aqueous methanol (30 ml), and 50% aqueous methanol (30 ml) at a constant flow rate of 1 ml/min. The eluent was collected at 1.0-minute intervals, and aliquots counted directly in 5 ml Instagel scintillation fluid (Packard Instruments).

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**Gas chromatography-mass spectrometry analysis using trimethylsilyl derivatization (Latif, S.A., Reinhold, V., Morris, D.J., unpublished data) has indicated that the major component of this material is a mono-hydroxylated metabolite of tetrahydroaldosterone. The position of the hydroxyl group in the steroid nucleus has not been determined. Alternative approaches to confirm this structure are now under investigation.

**Rat Mineralocorticoid Bioassay**

**Peak A Material**

All three dosages of peak A material (1, 5, and 25 µg) caused a significant decrease in the urinary Na+/K+ ratio in adrenalectomized male rats, the 5 and 25 µg dosage causing almost maximal effects (fig. 2 A). This polar aldosterone metabolite possessed 2% to 3.5% of the mineralocorticoid activity of aldosterone. As shown in figure 2 B and C, the magnitude of the...
antinatriuretic activity (Na⁺/creatinine) and kaliuretic activity (K⁺/creatinine) of the peak A material appeared to be similar, 2.0% to 3.5% that of aldosterone.

Reduced Metabolites of Aldosterone

All of the reduced derivatives exhibited some ability to lower the urinary ratio of Na⁺/K⁺ in the adrenalectomized male rat (fig. 3A). In all cases this resulted from a combination of antinatriuretic (fig. 3B) and kaliuretic (fig. 3C) properties. Differences in the overall activities between reduced compounds (5α-DHA > 3α, 5α-THA > 3β, 5α-THA > 5β-DHA 3β, 5β-THA) was largely caused by differences in antinatriuretic activity. Many of the derivatives, in particular, 5α-DHA and 3α, 5α-THA, appeared to be more active antinatriuretic than kaliuretic agents. 5α-DHA and 3α, 5α-THA possessed 1/10 and 1/30th of the antinatriuretic activity of aldosterone, respectively, whereas 3α, 5β-THA and 3β, 5α-THA possessed 1/100 and 1/200th of the antinatriuretic activity of aldosterone, respectively. The remaining 5β-metabolites were weaker agonists. The kaliuretic activities of the reduced compounds were very similar, and except for 3β, 5β-THA, they were all approximately 1/100th as active as aldosterone.

Angiotensin II Binding Studies

In preliminary experiments the material that eluted from HPLC columns at 60 minutes (peak A) inhibited binding of [125I]-angiotensin II to bovine adrenal glomerulosa cells. At a concentration of 10 μg/ml, it inhibited 36% of the binding that occurred when methanol alone was present, and at a concentration of 3 μg/ml, it inhibited 20%. Extrapolation from these figures, assuming that the metabolite had a molecular weight of 380, gave the ID₅₀ of the compound at about 3 × 10⁻⁵ M. Earlier eluates from the HPLC columns and aldosterone itself had little or no inhibitory effect on angiotensin binding.

Discussion

The major site for the metabolism of aldosterone in the rat, dog, and human is the liver. However, the kidney can also produce aldosterone glucuronides and small quantities of ring A-reduced metabolites of aldosterone. Earlier studies have shown that the specific reduced metabolites of aldosterone synthesized in the liver depend on the species, sex, and subcellular fraction of the tissues studied. The reduced metabolite, 3α, 5β-THA, is synthesized by liver cytosol, whereas 3β, 5α-THA is the major reduced metabolite synthe-
sized by microsomes from male rat liver, and 3α, 5α-THA by microsomes from female rat liver. We have shown a variety of polar neutral metabolites and reduced metabolites to be present in liver, kidney, and plasma soon after the administration of physiological quantities of 3H-aldosterone to male rats. In the present studies, the Kagawa mineralocorticoid bioassay (reduction in urinary Na+/K+ ratio) indicated the following decreasing potencies: aldosterone > 3α-DHA, > 3α, 5α-THA, > 3β, 5α-THA, > 3β-DHA, > 3β, 5β-THA. Of interest were the differences in the antinatriuretic and kaliuretic activities observed among the reduced metabolites. A clear hierarchy of antinatriuretic activities was observed and showed that 5α-DHA and 3α, 5α-THA possess 1/10th and 1/30th of the activity of aldosterone respectively, whereas 3α, 5β-THA and 3β, 5α-THA possess 1/100th and 1/200th the activity respectively. It appears that the 5α-compounds are more active than the 5β-configurations and likewise that 3α-compounds are more active than the 3β-products. The kaliuretic activities of all of the reduced metabolites were much lower than their antinatriuretic activities, possessing only 1/100th to 1/200th the activity of aldosterone. These findings not only provide further evidence that the antinatriuretic and kaliuretic events are separable, but also suggest that the 5α-reduced metabolites are more closely related to the antinatriuretic component of the physiological response.

FIGURE 3. The mineralocorticoid activities of the ring A-reduced metabolites of aldosterone in male adrenalectomized rats. A. Overall activity is expressed as a decrease in urinary Na+/K+. B. Antinatriuretic activity is expressed by a decrease in Na+/creatinine. C. Kaliuretic activity is expressed by an increase in K+/creatinine. Values are means ± se; n = 5 to 10 for each dosage.
The peak A material (fig. 1), which contains a monohydroxylated metabolite of tetrahydroaldosterone, was shown to possess similar antinatriuretic and kaliuretic activity, both approximately 1/30th–1/50th that of aldosterone. In addition to the liver, the kidney also synthesizes the peak A material. It has been shown to be present in vivo in plasma and kidney during the latent period of action of aldosterone. It is interesting, also, that peak A is the major metabolite excreted in the urine of adrenalectomized male rats during the first hour after the injection of aldosterone (during the latent period), whereas, normally, only small quantities of reduced metabolites are excreted in the urine of rats.19

Since the reduced metabolites and the peak A metabolite possess less activity than their parent compound, aldosterone, it is unlikely that the hepatic synthesis of these metabolites play a direct role in the expression of aldosterone activity in the kidney. However, it is possible that these metabolites are involved in other direct vascular or peripheral extrarenal actions of aldosterone20 and/or serve as substrates for further renal metabolism to other important products. The finding that the peak A material is both reduced and hydroxylated, and that the reduced metabolites are absent in vivo in urine, suggests that the reduced metabolites are part of a sequence of enzymatic products leading to the synthesis of hydroxylated metabolites.

A previously reported21 example of an extrarenal effect is the observation that steroid metabolites inhibit adrenal angiotensin receptors. The potency of the peak A material tested in the current study is greater than the potency of any other steroid so far studied. The physiological relevance of this in vitro observation is not known at this time, since the steroid's inhibitory potency is low relative to angiotensin itself.

In our most recent experiments,4 high resolution HPLC has resolved the reduced metabolites synthesized in the kidney into at least four of the six possible ring A-reduced metabolites. We have now shown that at least 60% of the reduced metabolites synthesized by kidney slices are 5α-reduced products. Approximately half of this co-chromatographs with the two standards, 5α-DHA and 3α, 5α-THA, both of which possess considerable antinatriuretic activity. 3α, 5β-THA, and 3β, 5α-THA (which are weaker mineralocorticoid agonists) are also synthesized in kidney. The synthesis of these metabolites as well as the polar neutral metabolites in the kidney suggests that the metabolism of aldosterone in situ in the kidney plays a role in the mechanism of action of aldosterone. The apparent lower mineralocorticoid activity of these metabolites observed when administered exogenously in the Kaga-wa bioassay system may be the result of reduced bioavailability and, therefore, may misrepresent their physiological importance. It is possible that an endogenous supply of these metabolites provided by renal synthesis close to the site(s) of mineralocorticoid action increases their relative potency and biological importance.

A finding that might support this hypothesis is that a significant proportion of the metabolism to both the polar and reduced metabolites in the kidney takes place

**Figure 4.** The possible role of aldosterone transformations in the expression of mineralocorticoid activity.
in purified nuclear and plasma membrane fractions. Significant quantities of these metabolites are also synthesized by renal cytosol and microsomal fractions. The antimineralocorticoids, spironolactone, and progesterone inhibit the synthesis of the reduced, as well as the polar, metabolites of aldosterone in kidney slices and nuclear and plasma membrane fractions.

Localization of these 5α- and 5β-pathways of aldosterone reduction and other routes of metabolism in nuclei places these transformations near the site of interaction of the hormone-receptor complex with chromatin, an important step in the mechanism of action of aldosterone.22-23 Thus, it is possible that some of the aldosterone transformations in the kidney are post-cytosolic receptor events, and the metabolites synthesized could have direct access to nuclear receptors. Aldosterone transformations by plasma membranes may also be of physiological importance since they would take place at sites close to Na\(^+\) and K\(^+\) transport. Although speculative, it would seem appropriate at this time to suggest that aldosterone transformations in target tissues may represent important additional steps (fig. 4) in the accepted mechanism of action of aldosterone.22-23 The ability of a target tissue to produce important metabolites may be a key factor in providing physiologically functional quantities to receptors localized in the same target tissue. Additionally extrarenal aldosterone transformations (in liver, etc.) may provide a supply of aldosterone metabolites that are biologically important or further transformed by the kidney and/or other target tissue.

At present, in vivo experiments have only shown the presence of aldosterone on the cytosolic receptor,22-24 whereas nuclei contain considerable quantities of aldosterone and its reduced and polar metabolites.1,2 Further experiments are therefore necessary to determine more about the relative rates of uptake of tritiated aldosterone and its reduced and polar metabolites by kidney slices, their rates of translocation to the kidney nuclei, as well as their relative affinities to both cytosolic and nuclear receptors.

The experiments outlined above suggest that further studies should be performed to explore the possible role of aldosterone transformations in the regulation of the renin-angiotensin system and Na\(^+\) and K\(^+\) homeostasis.

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