Aldosterone Esters and the Heart

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There are clinical and experimental situations in which symptoms of mineralocorticoid excess are remediable with mineralocorticoid receptor antagonist treatment, in spite of paradoxically low levels of plasma renin and aldosterone. Several decades ago, a factor isolated from the heart was described that had mineralocorticoid properties like those of aldosterone, but much more potent. It was thought to be similar to aldosterone-18-monoacetate or -21-monoacetate, acetyl derivatives of aldosterone that are very rapidly hydrolyzed in the circulation. In our efforts to confirm and extend these observations, we extracted rat hearts and plasma harvested in a manner that would minimize hydrolysis. The product was subjected to several forms of TLC and HPLC and compared to several acetylated derivatives of aldosterone standards. We found that 68% of the aldosterone extracted from fresh myocardium corresponded to an aldosterone derivative that migrates at the same rate as aldosterone-20-monoacetate. The identity of this compound awaits definitive analysis. Tritiated aldosterone-21-monoacetate hydrolyzed to form aldosterone very rapidly; negligible monoacetate remained in blood left at 37°C for 5 min or in hearts left at room temperature for 30 min. Regulation of aldosterone production serves the requirements of fluid and electrolyte homeostasis provided by transport epithelia, primarily that of the kidney. Nonepithelial actions of aldosterone would be freed of these regulatory constraints if the formation of a more potent derivative of the parent compound to which it is almost immediately hydrolyzed in the circulation were regulated within the nonepithelial target tissues. Am J Hypertens 2001;14:200S–205S © 2001 American Journal of Hypertension, Ltd.

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Aldosterone acts through high affinity intracellular mineralocorticoid receptors (MR), which act as transcription factors to mediate genomic effects, as well as by membrane receptors that mediate rapid nongenomic effects.1,2 Membrane receptors mediating nongenomic actions of mineralocorticoids upon the vasculature and the heart remain poorly characterized and have yet to be isolated or cloned. Genomic effects are better understood and occur both in transport epithelia and in nonepithelial tissues, including the heart, major vessels, and brain, where MR receptors are also widely distributed.5–7 The MR have been demonstrated in all four chambers of the heart using cytosol binding studies in vitro7 and in vivo.6 The ligand-bound MR is a transcription factor that regulates the expression of several cell-specific proteins mediating mineralocorticoid action.8 Among these are factors producing structural changes in the heart and vessels independent of mineralocorticoid effects on blood pressure.9–15

The mineralocorticoid aldosterone and the glucocorticoids corticosterone and cortisol are synthesized by a series of common enzymatic steps leading from cholesterol to deoxycorticosterone (DOC). The final steps in the synthesis of corticosterone and aldosterone are catalyzed by two closely related enzymes, 11β-hydroxylase and aldosterone synthase, products of the CYP11B1 and CYP11B2 genes, respectively.16,17 The DOC is converted by 11β-hydroxylase in the zona fasciculata reticularis to corticosterone and by aldosterone synthase in the zona glomerulosa successively to corticosterone, 18-hydroxycorticosterone, and aldosterone. In several species, including humans, the 17-hydroxylase in the zona fasciculata generates 11-deoxycortisol, converted by 11β-hydroxylase to cortisol.

The assumption that adrenocorticosteroid production is limited to the adrenal gland was challenged by the experimental demonstration of their extra-adrenal synthesis in the heart, large vessels, and brain. Synthesis of corticosterone and aldosterone has been shown in vascular smooth muscle cells,18 vascular endothelial cells,19 brain,20,21 and heart.22 Recent studies suggest that the regulated secretion of aldosterone in the heart might be involved in myocardial fibrosis.
dial remodeling after a myocardial infarction, in the spontaneously hypertensive rat, and in high salt diet-induced myocardial hypertrophy. The Dahl salt-sensitive rat has low plasma aldosterone, yet, paradoxically, their hypertension is easily controlled by mineralocorticoid receptor antagonists infused systemically or centrally. The intracerebroventricular infusion of a mechanism-based inhibitor of the aldosterone synthase enzyme demonstrated a role for the synthesis of aldosterone in the brain of the Dahl salt-sensitive rat in their hypertension.20 Because of the vanishingly small amounts produced even in optimal in vitro systems, it is certain that steroids produced extra-adrenally must act locally, if at all.

Studies done 30 years ago by Mary F. Lockett demonstrated that heart extracts contained a substance with antinatriuretic activity and physicochemical properties qualitatively similar to those of the 18-monoacetyl derivative of aldosterone.26,27 This substance produced the same physiologic effects, but was significantly more potent than aldosterone. An isolated perfused rat heart secreted decreasing quantities of the substance with time, the synthesis of which was increased by perfusion with media-containing aldosterone.28 Perfusion with 3H-aldosterone generated the labeled derivative. This “heart factor” was isolated from the hearts of cats, pigs, rats, and sheep.27,29 This area of study lay dormant for 30 years. We are reporting our studies on the isolation of a derivative of aldosterone from the rat heart that is less polar than aldosterone and very similar or identical to that reported by Knox and Lockett.27

**Methods**

Aldosterone, aldosterone 21-monoacetate, other steroids and reagents were obtained from Sigma Chemical Company (St. Louis, MO). Solvents and TLC plates were obtained from Fisher Scientific Company (St. Louis, MO). An aldosterone monoclonal antibody was raised against aldosterone-3-carboxymethoxime and used for an ELISA. A sheep antialdosterone-18,21-dissuccinate antibody was kindly provided by the National Institutes of Health and characterized by us.31 This antibody cannot distinguish between aldosterone and the esters, as they cross-react completely.

**Preparation of Aldosterone-18-Monoacetate and Aldosterone-20-Monoacetate**

Aldosterone can form 18-, 20-, and two different forms of 21-monoacetates, as shown in Fig. 1. Aldosterone-18-monoacetate was prepared as described by Knox and Lockett.27 The preparation of aldosterone-20-acetate was more difficult and required the formation of the aldosterone-20,21-diacetate using perchloric acid catalysis with acetic anhydride and careful hydrolysis using 1% sodium bicarbonate followed by chromatographic separation using a tapered TLC plate with chloroform:isopropanol 4% and developed three times. The final yield was around 2%. The material had chromatographic properties distinct from the other acetates and upon hydrolysis regenerated the initial aldosterone.

**Extraction of Rat Heart**

Two Sprague-Dawley rats were anesthetized with isoflurane/O2 anesthesia, left ventricular blood collected by cardiac puncture into iced vacutainers containing lithium heparin was centrifuged immediately at 2°C and the plasma extracted with ice-cold methanol. The hearts were removed and immediately homogenized in ice-cold methanol. The extracts of the hearts and 2 mL of plasma were centrifuged and evaporated at room temperature under flowing nitrogen. The solid extract was subjected to TLC chromatography using a 250-µm silica gel GF254 plate and developed using chloroform:methanol (95:5 v/v). Standards for aldosterone and aldosterone monoacetate were run in parallel, separated by two lanes from the sample. Aldosterone-21-oleate standard is considerably less polar than the acetates and was also run in a separate experiment. The lane immediately next to the sample was
used for the determination of blanks. The areas corresponding to aldosterone and the monoacetate were eluted with methanol, evaporated and subjected to RIA using the aldosterone-18,21-diacetate antibody. After the demonstration of immunoreactivity corresponding to the monoacetate, a new extract was made and the area corresponding to the monoacetate was hydrolyzed using potassium bicarbonate and assayed using an ELISA with the monoclonal antibody.

Extracts from four rat hearts were also subjected to HPLC using a C18 reverse phase column and eluted with 46% methanol in water. The column was eluted at a rate of 1 mL/min and 0.5-min aliquots were collected using a fraction collector. The fractions were then used for measuring aldosterone after hydrolysis using the aldosterone monoclonal antibody. This column cannot separate the various acetates.

An additional experiment was done in which the area corresponding to the aldosterone monoacetate by HPLC was evaporated and subjected to Florisil TLC and developed with 2% ethanol in chloroform. The plate was scrapped and the Florisil extracted with methanol, evaporated and subjected to RIA for aldosterone. Parallel running standards for the various acetates were used. The Rf for aldosterone-18-monoacetate was 0.28, aldosterone-21-monoacetate was 0.2, and aldosterone-20-monoacetate was 0.12.

Hydrolysis of Aldosterone-21-Monoacetate by Serum

Serum was incubated with 1 μCi of 3H-aldosterone-21-monoacetate at 37°C and 0°C for 5 and 30 min, extracted with dichloromethane, and subjected to TLC chromatography as described above. The hydrolysis was measured by scanning the TLC plate in a Bioscanner.

Results

The extraction of rat hearts with methanol, followed by separation by TLC and then RIA measurement indicated the presence of aldosterone and a compound that migrated at the same rate as aldosterone monoacetate. The concentration of aldosterone in the heart sample was 3 ng/g of which 68% was monoacetylated. The left ventricular plasma concentration was 0.28 ng/mL of immunoreactive material, 50% of which was monoacetylated. A portion of the monoacetylated aldosterone was hydrolyzed and aldosterone was measured using an ELISA with a highly specific antibody. A similar sample of heart extract was subjected to HPLC and the fractions measured using a RIA and ELISA. Fig. 2 shows the elution pattern of aldosterone and aldosterone monoacetate from the heart extract. Again the aldosterone monoacetate peak was significantly larger than free aldosterone. Attempts to increase the extraction yield by using large hearts was not successful. Rapid extraction of small pieces of equine heart provided similar monoacetates as that from rat hearts, but when a whole heart was minced, then extracted, very little acetate was obtained, suggesting that hydrolysis proceeds very rapidly once the tissue is disturbed and enzymes are released.

To distinguish between the different acetates, the monoacetate area obtained from TLC was subjected to a Florisil TLC, which can separate the three acetates. The immunoreactive area obtained corresponded to aldosterone-20-monoacetate (Fig. 3). Aldosterone-21-monoacetate added to blood was hydrolyzed completely within 5 min of incubation at 37°C and approximately 50% after 30 min at 0°C.

Discussion

Aldosterone in solution at physiologic pH and temperature exists as a mixture of the 11β-18-hemiacetal and the 11β-18-20-bicyclical acetal. In crystalline form, aldosterone only exists as the bicyclical acetal. These two isomers of aldosterone allow the formation of three different monoacetates, the 18-, 20-, and 21-monoacetates and the 21-monoacetate can form either of two isomeric forms, as shown in Fig. 1. The immunoreactive compound isolated from rat hearts migrates on a Florisil plate at the same rate as aldosterone-20-monoacetate, suggesting that the 20-monoacetate is the heart factor described by Lockett. This conclusion requires confirmation by structural analysis. In corroboration of results reported by Lockett and her co-workers, our as yet unpublished studies indicate that both aldosterone-18-monoacetate and -21-monooleate are more potent pressor agents than aldosterone when infused continuously intracerebroventricu-larly in rats. As acetate derivatives of aldosterone hydrolyze almost immediately in blood to form the parent compound, those produced in the heart would not persist under usual conditions.
procedures for obtaining blood even if an appropriate assay were used. Because passage through the lungs resulted in the disappearance of the monoacetate, direct arterial infusions were used to compare the potency of aldosterone and aldosterone-18-monoacetate in altering urinary electrolyte excretion.34

Until relatively recently left ventricular hypertrophy (LVH) was assumed to represent a maladaptive response of the heart to systemic hypertension, the primary risk factor associated with LVH. However, similar elevations of blood pressure result in a wide range of variations in the extent and type of hypertrophy.35 A linear relationship was demonstrated between plasma aldosterone levels and left ventricular hypertrophy in patients with moderate essential hypertension.36 Patients with primary aldosteronism have thicker intraventricular septi and posterior walls and a higher LV mass index compared to patients with similar elevations in blood pressure due to other forms of hypertension.10,12,37 Removal of the adrenal adenoma with normalization of aldosterone levels results in the reduction of LV mass in most primary aldosteronism patients37; however, medical treatment with an aldosterone antagonist did not.10 The incidence of primary aldosteronism is far more common than previously recognized, with an incidence of 7% to 12% in relatively unselected patients diagnosed as having essential hypertension.38–41 Most or all of the patients in these studies had no evidence of hypokalemia, the pathognomonic sign of excessive aldosterone action in the kidney.41 Spironolactone treatment is clearly beneficial in the treatment of congestive heart failure and decreases myocardial collagen turnover in human patients.42,43 Animal studies have provided clear evidence that mineralocorticoids produce cardiac hypertrophy and fibrosis independent of their hypertensinogenic effects.44,45 The adrenal cortex, but not the adrenal medulla, was necessary for the development of cardiac hypertrophy associated with aortic coarctation in rats, although the blood pressure increases produced by aortic coarctation in the adrenalectomized, medullectomized, and sham adrenalectomized animals were similar.46

There is also a significant proportion of patients with essential hypertension who respond to mineralocorticoid antagonists, although their plasma renin and aldosterone are low or normal and they are not hypokalemic.47,48 A similar phenomenon is seen in the Dahl salt-sensitive rat. This genetically hypertensive rat has normal-to-low serum levels of aldosterone that are further lowered by the high salt diet used to accelerate the development of hypertension. Yet the hypertension, cardiac hypertrophy, and renal failure the Dahl salt-sensitive rat experience are extraordinarily sensitive to treatment with mineralocorticoid antagonists.20,44 In another rat model, the spontaneously hypertensive stroke prone rat, which do not have high circulating aldosterone levels, but in which the renin-angiotensin system (RAS) system appears to be a major driver of the hypertension, the use of a mineralocorticoid receptor antagonist mitigated cardiac, cerebrovascular, and renal pathology, even in the severely hypertensive animals.9,49

The formation of a more potent acetyl derivative would amplify cardiac effects of relatively small increases in aldosterone. The concentration of aldosterone in myocardium was reported to be approximately 16 nmol/L, a value that is clearly higher than the mean concentration in plasma (~0.93 nmol/L).22,50 We obtained similar values, but found more than 70% of the aldosterone in fresh myocardium is in the fraction corresponding to the monoacetates. The mechanism for increased concentrations of aldosterone in myocardium and acetylation of aldosterone is not clear. It may be due to synthesis within the heart25 or from sequestration from the circulation, as has been shown in humans and rats.28 Expression of aldosterone synthase enzyme mRNA has not been found in the human heart.52

The regulation of aldosterone production by the adrenal glomerulosa is tied to its function in maintaining fluid and
electrolyte homeostasis provided by secretory epithelia, primarily that of the kidney. Secretion of aldosterone by perfused hearts in a Langendorf preparation was increased if the donor rats received chronic infusions of angiotensin II or a low sodium diet, suggesting similar regulatory mechanisms for the control of adrenal and extra-adrenal synthesis.\(^\text{22,24}\) However, another laboratory found that aldosterone synthesis in the perfused heart increased in rats given a chronic high sodium diet, whereas adrenal synthesis of aldosterone in these rats, as expected, decreased.\(^\text{25}\) If cardiac synthesis of aldosterone is physiologically relevant, it may be controlled by the cardiac RAS and be independent of the systemic RAS and the needs of the systemic circulation. Similarly, if formation of acyl derivatives of aldosterone that are more potent than aldosterone serves a physiologic function, one would assume that either the acylation or hydrolysis within the target organ would be regulated, but the mechanism is unknown. A permutation of aldosterone sequestration from the circulation or local synthesis, and potentiation of mineralocorticoid action by the formation of an ephemeral derivative plus regulation by an intracardiac system such as the cardiac RAS, would explain clinical and experimental evidence for mineralocorticoid action in the heart, which are not commensurate with circulating levels of this hormone.

Esterification of steroids by tissues and their presence in circulation has been amply demonstrated.\(^\text{53}\) Aldosterone has been shown to be esterified to an oleate and other esters in mammary gland tissue.\(^\text{54}\) We found that the esters in mammary gland tissue. We found that the has been shown to be esterified to an oleate and other oleate and comigrated with the 20-acetate. Definitive dem-

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