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Aldosterone and mineralocorticoid receptors: Orphan questions

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Aldosterone and mineralocorticoid receptors: Orphan questions. Classically, mineralocorticoid receptors (MR) are activated by aldosterone to promote unidirectional transepithelial sodium transport. Activation of MR in nonepithelial tissues has been shown to elevate blood pressure (central nervous system; CNS) and to cause hypertrophy and fibrosis (heart). For both epithelial and nonepithelial tissues, there remain a variety of questions regarding MR which are not only unanswered but also essentially not addressed. Seven such questions include: (*1*) how the physiologic glucocorticoids (cortisol and corticosterone) can mimic aldosterone action in epithelial MR, but act as antagonists in the heart and AV3V region; (*2*) how salt facilitates the nonepithelial, pathophysiologic effects of aldosterone; (*3*) how aldosterone activates unprotected AV3V MR in the face of orders of magnitude higher circulating glucocorticoid concentrations; (*4*) how unprotected nonepithelial MR act as "always occupied" receptors in guinea pigs and other species; (*5*) how, when 11β hydroxysteroid dehydrogenase type 2 is active, epithelial MR occupied by physiologic glucocorticoids appear transcriptionally inactive; (*6*) how aldosterone activates epithelial MR in the face of approximately 10^3-fold higher glucocorticoid levels, plasma binding and 11β hydroxysteroid dehydrogenase type 2 activity notwithstanding; and (*7*) how aldosterone produces changes in urinary $[K^+]$ before $[Na^+]$.

Seven is a magic number. We have seven days of the week and seven deadly sins; Europe is full of magic groves, from Sevenoaks to Sette Quercia. The Bible tells of seven lean years and seven fat years. Ingmar Bergman has the seventh seal, the Americans seven brides for seven brothers, the musical scale seven notes, in Arabic from gam to ut. One seventh is 0.142857, two sevenths 0.285714, three sevenths 0.428571, and so on. If you sailed the seven seas, you may meet the seven sisters and be in seventh heaven. Seven wonders of the world, seven pillars of wisdom, seven dwarfs.

There are a number of points that need to be made as background for a discussion of the seven questions posed. Mineralocorticoid receptors (MR) are members of a superfamily of steroid/thyroid/retinoid/orphan (STRO) receptors; their closest relatives are receptors for glucocorticoids, progestins, and androgens (GR, PR, and AR). STRO receptors are intracellular and operate (STR) as ligand-activated transcription factors to regulate gene expression. Such receptors have a domain structure, with the highest degree of receptor identity (≥90% for MR, GR, PR, and AR) in the DNA-binding domain (DBD) and substantial homology (≥50% for MR, GR, PR, and AR) in the ligand-binding domain. Orphan receptors may have no ligands or as yet undiscovered ligands: we knew a lot about opiate receptors before the discovery of the endogenous opioid peptides.

The human MR (hMR) is a 984 amino acid protein, for which the cDNA was cloned and sequenced by Arriza et al over a decade ago [1]. In this landmark study two of the central paradoxes of MR were addressed, recapitulating previous studies in rat tissue extracts [2]. First, when rat tissue extracts were probed with cDNA for hMR, receptor expression was found in kidney and gut as anticipated, but at much higher levels in the hippocampus, clearly a nonepithelial tissue. Secondly, when recombinant hMR are expressed in COS cells [1], or tissue preparations from adrenalectomized rats [2, 3] or guinea pigs [3] used as a receptor source, a challenging pattern of affinity for corticosteroids is seen: MR bind aldosterone and cortisol with indistinguishable, high affinity, and corticosterone with a slightly higher affinity. The first question raised by these studies is that of the physiological role(s) of nonepithelial MR in tissues such as the CNS and heart; the second is how aldosterone can ever occupy MR in epithelia, where it clearly can and does act, given the equivalent or higher affinity of the physiologic glucocorticoids for such receptors, and their much higher circulating concentrations.

In terms of the second of these questions, the answer appears to lie in the operation in epithelial aldosterone target tissues of the enzyme 11β hydroxysteroid dehydrogenase type 2 (11βHSD2). The enzyme was simultaneously cloned from sheep and human cDNA libraries; perhaps counterintuitively, the sheep studies emanated from the United States [4] and the human studies from

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Australia [5]. Its operation as a gatekeeper conferring aldosterone specificity on nonselective MR had been previously proposed on the basis of experimental studies in rats [6, 7], which, in turn, were based on the syndrome of apparent mineralocorticoid excess (AME). AME was first described over 20 years ago as a syndrome of severe juvenile sodium retention and hypertension in the face of normal or suppressed plasma levels of renin and aldosterone [8]; its etiology has subsequently been shown to reflect loss-of-function mutations in the gene coding for 11βHSD2 [9].

11βHSD2 functions as a gatekeeper because, unlike MR, it can discriminate between aldosterone and the physiologic glucocorticoids. For the latter, the enzyme is operationally unidirectional, converting glucocorticoids to their inactive 11-keto congeners, cortisone and 11-dehydrocorticosterone. Both of the latter have much lower affinity for MR (and GR), and if they do occupy MR act as antagonists. In the zona glomerulosa of the adrenal gland, the enzyme aldosterone synthase (CYP11B2) converts the methyl (CH_3) group at carbon 18 of the steroid molecule to an aldehyde (CHO) group, from whence comes the name aldosterone. In solution, this highly reactive aldehyde group cyclizes with the hydroxyl at carbon 11 to form an 11,18 hemiacetal and to make aldosterone not a substrate for 11βHSD2.

Upon this very brief background sketch of MR and 11βHSD2, the seven questions will be put, as follows.

QUESTION 1

How is it that the physiologic glucocorticoids (cortisol, corticosterone) mimic aldosterone in epithelial mineralocorticoid receptors, but act as antagonists in the heart and the AV3V region?

The syndrome of AME, as noted above, is characterized by inappropriate and very marked Na^+ retention in response to the inability of the loss-of-function mutant 11βHSD2 to metabolize cortisol, and thus exclude it from renal and colonic MR. As this suggests, cortisol in epithelial MR acts to mimic aldosterone. When 11βHSD2 is blocked by carbenoxolone, the hemisuccinate of glycyrrhetinic acid, corticosterone similarly can act as a potent mineralocorticoid in Kagawa bioassays in the adrenalectomized rat, in the presence of excess RU486 to exclude the corticosterone from GR [10]. What these Kagawa bioassay studies also show is that GR occupancy in epithelial aldosterone target tissues is similarly followed by a mineralocorticoid response, in that the highly selective GR agonist RU28362 in the presence of carbenoxolone produces an electrolyte response equivalent to that of aldosterone. In more recent studies [11], dexamethasone has been used in A6 cells to induce a classic "mineralocorticoid" response, induction of sgk synthesis (discussed later in this article), further evidence for the lack of selectivity at the nuclear response-element level, and the pivotal specificity-conferring role for 11βHSD2 in epithelia.

In extraepithelial tissues, 11βHSD2 is not coexpressed with MR, which are thus putatively "unprotected" and overwhelmingly occupied by cortisol/corticosterone in vivo. In addition, in a variety of experimental studies, occupancy of such MR by the physiologic glucocorticoids does not mimic the effects of aldosterone, but antagonizes them. Intracerebroventricular infusion of minute doses of aldosterone (10 ng/h), for example, has been shown to elevate blood pressure in the rat over a time course of days or weeks; intracerebroventricular infusion of corticosterone (20 ng/h) alone has no effect on blood pressure, but when coadministered with aldosterone corticosterone very substantially blocks its hypertensive effects [12].

Second, a peripheral infusion of robust doses of aldosterone (0.75 μg/h) to rats is followed by hypertension, cardiac hypertrophy, and cardiac fibrosis, which are similarly substantially blocked by the coadministration of corticosterone at a 30-fold excess [13]; administration of even higher doses of corticosterone alone does not cause hypertension, cardiac fibrosis, or cardiac hypertrophy [14]. In studies on isolated neonatal rat cardiomyocytes, a similar distinction between MR occupancy by aldosterone and corticosterone can be shown, in that aldosterone increases [^{3}H]leucine incorporation into protein, whereas corticosterone does not [15].

There is thus a clear difference between how the physiologic glucocorticoids act in epithelial MR, as aldosterone mimics, and in nonepithelial tissues, where they act as antagonists just like spironolactone or RU28318. There is no difference between MR in different tissues in terms of sequence; a one- to two-fraction difference in a fast protein liquid chromatography (FPLC) elution profile has been shown for hippocampal and renal MR, consistent with there being an approximately 15K molecule bound to one but not the other receptor [16]. Apart from this study, which is now over a decade old, there appear to be no data on the tissue-specific factors that engender the difference in behavior of epithelial and nonepithelial MR occupied by corticosterone/cortisol. Whether the same mechanisms obtain in nonepithelial cells and in epithelial cells in which 11βOHSD2 is operant (question 5, below) to preclude an MR-glucocorticoid complex from transcriptional activity remains to be determined.

QUESTION 2

How does salt facilitate the nonepithelial, pathophysiological effects of aldosterone?

It has long been known in studies on experimental hypertension that uninephrectomy and obligate drinking of 0.9% NaCl solution sensitize rats to exogenous miner-

alocorticoid administration. In their pioneer studies on cardiac hypertrophy and fibrosis in response to aldosterone, Brilla and Weber showed that although uninephrectomized rats drinking 0.9% NaCl responded to peripheral infusion of aldosterone of 0.75 μg/h by developing cardiac hypertrophy and both perivascular and interstitial fibrosis, those on a restricted sodium intake did not [17]. In both situations, aldosterone may be anticipated to occupy MR to a similar extent, and the equivalent right and left ventricular fibrosis [14, 17] is strong evidence for a humoral rather than a hemodynamic etiology for the observed effects.

One possible way in which the high Na^+ status may determine the response to aldosterone, to synergize in producing hypertrophy and fibrosis, is suggested by a recent report from Gu et al [18]. In these studies, a 4% increase in ambient [Na^+] in vitro (from 146 to 152 mEq/L) was followed by very marked changes in various parameters measured in neonatal rat myocardial myoblasts and vascular smooth muscle cells. Whereas for both cell types cell diameter increased approximately 9% and cell volume approximately 27%, cellular protein content increased by approximately 50%, and the rate of protein concentration as gauged by [^{3}H]leucine incorporation almost doubled. The conclusion drawn by these authors is that Na^+ may thus have a direct effect to induce cellular hypertrophy in both heart and vasculature; the way in which such effects may be transduced into cardiac fibrosis remains to be explored.

QUESTION 3

How can aldosterone activate unprotected AV3V mineralocorticoid receptors in the face of orders of magnitude higher circulating glucocorticoid concentrations?

Whereas in epithelia MR and 11βHSD2 are closely colocalized—in kidney collecting tubule, colon, salivary gland duct cells, and sweat gland—the enzyme distribution in the brain is quite different. In the rat brain, on in situ hybridization, 11βHSD2 appears to be expressed in the commissural position of the nucleus tractus solitarius and the ventrolateral ventromedial hypothalamus. In addition, scattered labeled cells are found in the medial vestibular nucleus [19]. This distribution is quite distinct from that of 11βHSD1 in the brain, and also from that of MR; the latter are most heavily concentrated in the hippocampal and septal regions, and are also found in the AV3V region where they influence blood pressure regulation.

In both of these areas, unprotected MR are presumably overwhelmingly occupied by the physiological glucocorticoids, given their much higher circulating concentrations. That such AV3V MR can be occupied, despite this concentration difference, by circulating aldosterone is shown by the results of studies by Gomez-Sanchez, Fort, and Thwaites in JR/S (salt-sensitive) rats [20]. This strain of rat responds to a 6% NaCl intake by an increase in blood pressure over the subsequent days and weeks. If the rats are simultaneously infused intracerebroventricularly with the MR antagonist RU28318, blood pressure does not rise; if the RU28318 infusion begins during the period of salt-induced blood pressure rise it is attenuated, and blood pressure returns toward normal.

There are a number of points to be made from this study. First, even in the face of a 6% Na^+ intake aldosterone levels appear still sufficiently high to occupy at least some part of the AV3V MR population: evolution has been much more concerned with increasing aldosterone levels in the face of sodium deficiency or loss than in suppressing them in the face of a high salt intake. Second, unless there are non-11βHSD2 mechanisms for excluding glucocorticoids, only a tiny proportion of AV3V MR would seem to need to be occupied by aldosterone to elevate blood pressure. Third, such a non-11βHSD2 mechanism for excluding glucocorticoids appears unlikely, given the ability of corticosterone coinfused into the lateral ventricle to block the hypertensinogenic effect of centrally administered aldosterone. While no answer to this question is immediately obvious, two things would seem to be the case: (*1*) that the concept of "spare receptors" applies, so that only a tiny proportion of AV3V MR needs be occupied by aldosterone to produce hypertension; and (*2*) that even to achieve such low levels of occupancy aldosterone can probably access unprotected MR only during the nadir(s) of plasma glucocorticoid levels.

QUESTION 4

How can unprotected nonepithelial mineralocorticoid receptors act as "always occupied" receptors, in guinea pigs and other species?

As noted above, unprotected MR are prima facie constitutively occupied by physiological glucocorticoids in the CNS and elsewhere, given their high affinity and the high circulating glucocorticoid levels. Even if for species like humans or the rat occupancy may fall to levels between 90 and 95% at nadir glucocorticoid levels, such would not appear to be the case in the guinea pig, for the following reasons. The guinea pig has long been known to be corticoresistant, despite very high circulating levels of cortisol and modest levels of corticosteroid-binding protein (CBG), so that in contrast with other species only approximately 30% of cortisol is transcortin bound. Underlying this glucocorticoid resistance appear to be very low-affinity GR [21], with a frequency of mutation in the ligand-binding domain of the GR twice that predicted from mutation frequency elsewhere in the molecule [22]. Despite the very high total and free

cortisol levels, the affinity of guinea pig MR for aldosterone, corticosterone, and cortisol is identical to that in the rat—in kidney, colon, heart, and hippocampus [3].

The implication for unprotected MR of this study is profound. Rather than the dynamic range of unprotected MR occupancy by glucocorticoids being in the range of 90 to 99%, it is in the range of 99 to 99.9%, to a first approximation. The driving mutation in guinea pigs appears to be an Ala24Pro substitution in the otherwise invariant sequence of adrenocorticotropic hormone (ACTH), leading to a superagonist ACTH [23] and thus presumably favoring the continuing evolution of low-affinity/low-activity GR. In contrast, the MR appears pristine, and to have retained the high affinity found in species with much lower circulating levels of total and free GR.

We are unused to the concept of an "always occupied" receptor, although intuitively there is nothing particular about a constitutively inhibited system that responds to lifting of that inhibition over a relatively small albeit linear dynamic range. A second possibility is that of the formation of MR:GR heterodimers, of varying transcriptional efficiency compared with homodimers. Finally, MR have equivalent high affinity not only for corticosterone, cortisol, and aldosterone, but also for progesterone [24]. There are occasions, in the luteal phase, in pregnancy, and in the fetus, when circulating levels of progesterone are sufficiently high prima facie to compete with cortisol/corticosterone for binding to unprotected MR, in the CNS and elsewhere.

QUESTION 5

How is it that when 11βHSD2 is active epithelial mineralocorticoid receptors occupied by glucocorticoids appear to be transcriptionally inactive?

At a first approximation, there are approximately 10,000 hMR per principal cell in the collecting tubule, with an affinity for aldosterone and cortisol of approximately 1 nmol/L. In contrast, the numbers of h11βHSD2 molecules are much higher, of the order of 3 to 4 million per cell; their "affinity" for cortisol is, however, much lower than that of the receptor, as denoted by a K_m of 40 to 50 nmol/L. If access to both receptor and enzyme is competitive, then approximately 10% of cortisol molecules to be bound within the cell will be to MR and approximately 90% to 11βHSD2; that is, there will be a "leak" past the protective enzyme of approximately 10%.

This need not be the case if there are differences in intracellular access between MR and 11βHSD2 in terms of their availability to cortisol. If, for example, the 300- to 400-fold excess of 11βHSD2 molecules were arranged as a multimeric protective lattice around MR, particularly if such a lattice were several layers deep, the levels of free cortisol in the immediate physical environment of MR might be one thousandth of those in the cell as a whole. Under such circumstances, 11βHSD2 would be acting as a physical "protector" of epithelial MR, as well as a one-way cortisol sink.

That such a mechanism does not in fact operate is suggested by a series of in vivo studies in which adrenalectomized rats were injected in vivo with [^{3}H]aldosterone, alone or together with 1-, 3-, 10- to 100-fold nonradioactive aldosterone or corticosterone, and specific binding determined in kidney, colon, heart, and hippocampus [25]. As noted above, corticosterone has approximately three times the affinity of aldosterone for MR, and much higher affinity for plasma CBG (transcortin), such that whereas approximately 50% of plasma aldosterone is protein bound (to albumin) ≥95% of plasma corticosterone is bound to CBG and albumin. If there are no barriers to cellular or intracellular access of plasma-free steroid to MR, then nonradioactive aldosterone would be predicted to be approximately three times as potent as corticosterone in competing for MR.

In the heart this proved to be the case, but not in the three other tissues examined. In hippocampus, corticosterone proved to be approximately twice as good a competitor as nonradioactive aldosterone, due to the much higher reflection coefficient of the blood brain barrier for aldosterone. In the kidney and colon, in contrast with both nonepithelial tissues, 11βHSD2 operated to lower the observed ability of corticosterone to compete for [^{3}H]aldosterone binding to MR. In both tissues, nonradioactive aldosterone was 20 to 50 times as potent of competitor as corticosterone; that is, on average, 11βHSD2 adds an additional order of magnitude of steroid selectivity to MR, equivalent to the level of specificity conferred by the preferential binding of corticosterone by CBG, and very much in line with the approximately 10% "leak" predicted previously in this article. The plasma total concentrations of cortisol are characteristically approximately three orders of magnitude higher than those of aldosterone, so even with a tenfold advantage due to CBG, and another tenfold advantage due to 11βHSD2, most MR will be largely occupied by cortisol even in epithelial tissues. How is it that under such conditions we do not retain sodium and become hypertensive, as happens when cortisol occupancy of MR follows inhibition or congenital absence of 11βHSD2?

One possible answer may lie in the cosubstrate for 11βHSD2, NAD. If in the presence of NADH MR·cortisol complexes were transcriptionally inactive in epithelial tissues (as appears normally to be the case in nonepithelial tissues), then the apparent paradox would be explained. If this proves to be the case (and the crucial importance of redox state for GR-mediated transactivation has recently been shown in a series of elegant studies) [26, 27], then 11βHSD2 will have two interlinked, crucial roles: to metabolize cortisol to the low affinity,

antagonist product cortisone and to provide a particular intracellular milieu in which cortisol, which does bind MR, is transcriptionally inactive (presumably in contrast with aldosterone bound to MR). Whether this proves to be the case is susceptible to experimental determination: if so, such a redox-dependent activation/inactivation status may also be of relevance in nonepithelial tissues, allowing cortisol·GR complexes to act as sensors of intracellular redox status.

QUESTION 6

How can aldosterone activate epithelial MR, in the face of approximately 10^3-fold higher glucocorticoid levels, plasma binding and 11βHSD2 activity notwithstanding?

This is the other side of the coin to the previous question, which addressed how MR·cortisol complexes do not appear to be transcriptionally active. One possibility whereby aldosterone may be able to access epithelial MR is in the nadir of plasma GR concentrations, when experimentally it has been shown by sufficiently frequent sampling [28] that plasma corticosterone in rats may fall below detection limits. We are comfortable with the concept of pulsatile or episodic secretion of hormones [for example, luteinizing hormone–releasing hormone (LHRH)]; we are less used to the possibility of episodic action, when levels of a potential competitor for receptor binding fall episodically below a threshold level.

One possible additional outcome of such a system is aldosterone·MR-mediated responses to stress. When rats were stressed in the incremental phase of ACTH/corticosterone secretion, the amplitude of the corticosterone pulse increased, but the length of the nadir between the exaggerated pulse and that subsequent lengthened considerably. If aldosterone can occupy MR only at the nadir of the interpulse interval, stress may very much potentiate MR·aldosterone action in epithelial and/or nonepithelial tissues, by considerably enlarging the window of opportunity for receptor occupancy.

QUESTION 7

How can aldosterone produce changes in urinary $[K^+]$ before $[Na^+]$?

In 1960, Jean Crabbé defined a mineralocorticoid as a hormone that stimulated unidirectional, transepithelial Na^+ transport [29]. The first clearly aldosterone-induced protein to be cloned and characterized, sgk, clearly activates Na^+ transport; when coexpressed with the potassium channel ROMK in *Xenopus* oocytes, it had no effect, whereas coexpressed with α, β, and γ subunits of epithelial sodium channel (ENaC) sgk increased sodium flux sevenfold [11, 30]. What is of particular interest in this context, however, is that sgk is only one of three aldosterone-induced proteins cloned by Pearce et al from A6 cells, and the nature of these (and other possible induced proteins not identified to date) is at present unknown.

There is some evidence from in vivo studies that one of this group may provide a mechanism for aldosterone-induced K^+ flux, with a time course of action discernibly faster than that of sgk to increase the open probability of ENaC. In recent Kagawa bioassay studies on the urinary response of adrenalectomized rats to aldosterone (unpublished data), we measured urinary $[Na^+]$ and $[K^+]$ before steroid administration and at 30-minute intervals thereafter. In such studies, urinary $[Na^+]$ did not change over the first hour after aldosterone, whereas urinary $[K^+]$ rose progressively to be substantially (approximately 60%) and significantly higher in the +30- to +60-minute collection than control. A specific effect of aldosterone on $[K^+]$ excretion is not perhaps unexpected, given how potent quite small elevations in ambient plasma $[K^+]$ are in terms of increasing aldosterone secretion rate [31]. The mechanism(s) underlying this effect of aldosterone on $[K^+]$ excretion at a molecular level remains to be explored.

In summary, although there have been considerable recent advances in our understanding of how aldosterone acts in $[Na^+]$ transporting epithelia, a range of questions remain to be answered in both epithelial and nonepithelial tissue about roles for aldosterone, and more generally for MR. These questions may be answered without being directly addressed, as has on a number of occasions proved to be the case in other areas in which molecular biological techniques have been applied in endocrinology. On the other hand, some of them are susceptible to being directly addressed; and if this paper provides a stimulus for such inquiry, it will have served its purpose well.

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