Perspective

Adrenal Steroids: New Answers, New Questions

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WHEN THE PERSON IN THE STREET THINKS OF STEROIDS, what comes to his or her mind are oral contraceptives or pill-popping athletes. To the endocrinologist, however, steroid hormones are a family of information molecules, cholesterol-derived, and serving what at times seems a puzzling selection of physiological functions. Six classes of steroid hormones have been distinguished in mammalian systems, and a seventh (ecdysteroids) in invertebrates.

Although we tend to think of steroids as products of the ovary, testis, and adrenal glands, they are synthesized in a wide variety of tissues. The calcium-active steroid 1,25-dihydroxycholecalciferol (vitamin D_3) is synthesized by a sequence of processes in skin, liver, and kidney. In men, and in women before puberty or after the menopause, most estrogen is derived from circulating androgens by aromatization in fat, white blood cells, and other tissues. In the pregnant woman, progesterone is largely of placental origin.

In contrast, mineralocorticoids and glucocorticoids are produced only in the adrenal gland. Aldosterone is the principal physiological mineralocorticoid, from bony fish on up; in most species, cortisol (hydrocortisone) is the principal glucocorticoid, although in rats and mice the principal glucocorticoid is corticosterone.

Aldosterone synthesis and secretion is stimulated by angiotensin, produced by the action of the enzyme renin from the kidney on a circulating substrate, angiotensinogen. Renin secretion rises in response to sodium ion (Na^+) loss or deprivation; as befits a classical homeostatic hormone, aldosterone acts on epithelia (renal tubule, gut, salivary gland) to retain Na^+, by increasing unidirectional transepithelial Na^+ transport. Other physiologically coordinate aldosterone-specific responses (increases in blood pressure and salt appetite) have been reported in response to injection of the hormone into the cerebral ventricles.

In contrast, no such coherent physiology can yet be written for glucocorticoids. Their synthesis and secretion are controlled by adrenocorticotropin (ACTH) from the anterior pituitary gland, which in turn is responsive to the hypothalamus and other brain areas. In a classic negative feedback loop, glucocorticoids reduce ACTH synthesis and secretion, both by direct pituitary effects and by reducing the levels of hypothalamic trophic factors.

Here, however, the comfortable parallelism with mineralocorticoids stops. Glucocorticoids have effects on development, on cell replication, on metabolism, and on the expression of a diversity of genes in essentially all tissues of the body. ACTH and glucocorticoids rise in response to moderate-to-severe stress (for example, 20 minutes at 85 percent maximum when a subject is on a treadmill). What is often forgotten, in terms of glucocorticoid physiology, is that ACTH and glucocorticoid rise and fall on a circadian basis. In some species, the peak to nadir ratio can differ by a factor of 50; the implications of this—for development, cell replication, metabolism, gene expression—are rarely considered.

Fifteen years ago, preliminary studies from the laboratories of Gordon Tompkins and Isidore Edelman distinguished two sorts of high-affinity intracellular binding sites for adrenal steroid hormones (*1*). One class of site in rat kidney extracts had high affinity for aldosterone, apparently lower affinity for corticosterone, and lower affinity for the synthetic, non-salt-retaining glucocorticoid dexamethasone. These putative mineralocorticoid receptors were termed type I aldosterone binding sites. Type II sites were subsequently shown to be classical glucocorticoid receptors with substantially higher affinity for the potent synthetic steroids dexamethasone and triamcinolone acetonide than for corticosterone or aldosterone.

Ten years ago, Bruce McEwen and his colleagues described a second type of putative glucocorticoid receptor in rat hippocampus, called "corticosterone-preferring sites" since they have a higher affinity for corticosterone than dexamethasone (*2*). Subsequently, work from Ron de Kloet's laboratory has established that such sites are indeed physiological high-affinity glucocorticoid receptors (*3*). Adrenalectomy in rats produces changes in serotonin and serotonin receptor levels in the hippocampus; these changes are reversed by corticosterone, but not by equivalent doses of dexamethasone, indicating an action through the corticosterone-preferring sites, rather than through classical dexamethasone-binding glucocorticoid receptors.

Four years ago, work from several laboratories provided strong support for the identity of renal mineralocorticoid receptors and hippocampal corticosterone-preferring sites in cytosol preparations in vitro (*4*). In terms of steroid specificity, the receptor populations were indistinguishable, provided that steroid binding to other proteins (most notably transcortin, the plasma corticosteroid-binding globulin) was excluded. Under such conditions, the sites (now generically termed type I receptors) bind aldosterone and corticosterone with equal, high affinity, and bind dexamethasone with lower affinity. In addition, isoelectric focusing studies showed identical patterns on tryptic digestion of renal and hippocampal aldosterone-binding sites, clearly distinguishable from those of classical glucocorticoid receptors (*5*).

From these studies, then, two new conundrums arose for adrenal steroid physiologists. First, type I sites have equivalent affinity for aldosterone and corticosterone; given the much higher circulating free levels of glucocorticoids, how does aldosterone get its message through to mineralocorticoid target tissues such as the kidney? Second, given the very much higher affinity of type I sites for corticosterone or cortisol compared to type II sites, what are the physiological implications of having two sorts of glucocorticoid receptors, with substantially (at least an order of magnitude) different affinity for the same signal?

It is in this rather exhaustively presented context that the studies from Ron Evans' laboratory in this issue of *Science* (*6*) need to be appreciated. Eighteen months ago he and his colleagues published (*7*) the first full-length steroid receptor sequence, that of the human glucocorticoid receptor (hGR). At that time, they noted a homology between the v-erbA oncogene and the sequence coding for the cysteine-rich midportion of the hGR, a homology subsequently noted for other steroid receptors. Six months ago, the Evans group showed, back-to-back with a group of European investigators, that the human c-erbA oncogene product was the thyroid hormone receptor (T_3R) (*8*). And now the laboratory has landed the treble, by cloning, sequencing, and expressing the human kidney mineralocorticoid receptor (hMR).

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There are a number of things that need to be said. First, cloning, sequencing, and expressing one receptor is a substantial achievement; to have succeeded with three is a tour de force of molecular endocrinology, reminiscent of the success of Numa and his group with the three precursors for endogenous opioid peptides (*9*). Second, the cloning strategies used, for hT_3R and even more so for hMR, are elegant examples of logic in science. Finally, although the cloning hGR and hMR will obviously provide superb tools for further studies, the initial transcriptional activation experiments reported in the present article raise more questions than they provide answers.

► There is now very compelling evidence that steroid receptors and the thyroxine receptors—since there appear to be at least two hT_3R's, a neural and a hepatic type, encoded by genes on different chromosomes—are members of a receptor-oncogene superfamily. Each receptor consists of a COOH-terminal hormone binding region, a midportion cysteine-rich DNA-binding region, and an immunogenic NH_2-terminal region presumably involved in the initiation of transcription. Between receptors, the homology is highest in the midportion, intermediate in the steroid-binding domain, and low in the NH_2-terminus.

As Arriza *et al.* (*6*) point out, the high homology between hGR, hMR, and rabbit progesterone receptor (PR) sequences suggest that there may be a subfamily within this superfamily. The existence of at least two T_3R's, with the neural species showing even higher homology with the v-erbA oncogene product than the hepatic species, is evidence for a second subfamily. Between the two subfamilies (T_3R; GR, MR, PR) and the other steroid receptors for which partial or complete sequences are known (estrogen, ER; vitamin D_3, VDR) the extent of homology is 40 to 50 percent in the DNA-binding region, and about 20 percent in the COOH terminus (*10*). At least in the DNA-binding region, the homology is slightly higher within the group T_3R-ER-VDR than between this group and GR-MR-PR.

In the fullness of time, this superfamily scheme will probably need to be revised, and certainly extended, to include the androgen receptor; the E75 receptor from drosophila which may bind ecdysteroids, or possibly the sesquiterpene juvenile hormone; the dioxin "receptor" which appears to have considerable behavioral similarity to steroid receptors; and a number of open reading frame steroid receptor–like sequences which have been cloned and sequenced, but for which ligands are as yet unidentified.

► In the initial studies establishing steroid receptor sequences (GR, ER, PR), antibodies to more or less purified receptor preparations were used to screen appropriate complementary DNA (cDNA) libraries in expression vectors. For the T_3R, the Evans group exploited their finding of the 52 percent homology within the nuclear-binding regions of v-erbA and the hGR. Reasoning that the cellular counterpart of the v-erbA oncogene product may be a nuclear bound receptor, they screened human placental cDNA libraries with a v-erbA probe. The cDNA to which the probe hybridized was then transcribed and translated in vitro, and shown to bind T_3, establishing it as the thyroxine receptor without the intervening process of purifying receptors and raising antibodies.

The mineralocorticoid receptor has been notoriously unstable, particularly in broken cell preparations, and attempts to purify it have been uniformly disappointing. The "piggyback" approach used by Arriza *et al.* (*6*) is thus both conceptually elegant and practically necessary, given the difficulties of the classical, purification-antibody approach.

In this instance the initial step was to probe human genomic DNA under low-stringency conditions with a fragment of hGR cDNA containing the sequence coding for the DNA-binding domain. After Hind III digestion, a 2.5-kilobase fragment was found to hybridize with the probe. Accordingly, human placental DNA was digested with Hind III, size-fractionated, and the 2.5-kb region expanded into a subgenomic library. This library was then rescreened with the hGR cDNA fragment under low stringency conditions, and a positive clone was identified. This genomic clone, thus purified from other 2.5-kb Hind III fragments, was then used to screen human kidney cDNA libraries; hybridizing clones from λgt10 libraries were used to establish the putative hMR sequence, which was confirmed by binding studies after the gene was expressed in transfected cells.

► These experiments also provide the first direct evidence that the hMR is a transcriptional regulatory protein; yet, in many senses the transcriptional activation studies raise more questions than they answer. In two studies, receptor-negative cells were cotransfected with a construct called MMTV-LTR-CAT shown previously to respond to glucocorticoids in the presence of glucocorticoid receptors, and a plasmid expressing either hGR or hMR. Dexamethasone activated transcription of the reporter gene when either receptor was present: aldosterone in the presence of hMR, but not hGR.

At one level, it is hardly surprising that either receptor can bind to the glucocorticoid regulatory elements on MMTV-LTR, given the 94 percent homology in the DNA-binding region between hGR and hMR. From then on, however, the findings are less able to be predicted from in vivo physiology or whole cell receptor-effector studies. In classic glucocorticoid test systems, for example, aldosterone has moderate affinity for GR, but acts as a full agonist, for example, optimally inducing tyrosine aminotransferase in cultured hepatoma cells. In addition, dexamethasone in mammalian systems has vanishingly low if any activity as a mineralocorticoid agonist: its activity in the presence of hMR is therefore perhaps even more surprising than the failure of aldosterone to activate hGR.

It is the consolation of the in vivo physiologist, faced with perturbing data from in vitro studies, to reflect that such studies demonstrate what the system in question can do, not what it actually does. This bias declared, I would venture to predict that, when authentic mammalian genomic fragments are fused to reporter sequences and studies done with receptors cotransfected to yield normal cellular levels, the transcriptional activation obtained may parallel that found in classical specificity studies.

Finally, studies of this sort may eventually provide answers to two of the big questions that remain in the area of adrenal steroid physiology—how aldosterone occupies type I receptors in mineralocorticoid target tissues in the face of much higher glucocorticoid concentrations, and the implications of both high-affinity (type I) and low-affinity (type II) receptors for glucocorticoids, particularly in the central nervous system. In this latter context, the cloning of the mineralocorticoid receptor, long the Cinderella of the steroid receptor field, may prove to have much more profound implications for neurobiology than for salt and water homeostasis.

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