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Baker, Michael E

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Co-Evolution of Steroidogenic and Steroid-Inactivating Enzymes and Adrenal and Sex Steroid Receptors

Michael E. Baker

Department of Medicine, 0693

University of California, San Diego

9500 Gilman Drive

La Jolla, CA 92093-0693

phone: 858-858-534,-8317; fax: 858-822-0873

e-mail: mbaker@ucsd.edu

Abstract. Receptors for the adrenal and sex steroids arose by a series of gene duplications from an ancestral nuclear receptor in a primitive vertebrate, at least 540 million years ago. Sequence analysis indicates many steroidogenic and steroid-inactivating enzymes, including cytochrome P450s and hydroxysteroid dehydrogenases, arose at the same time. The estrogen receptor (ER) appears to be the ancestral steroid receptor. Initially, the redundant duplicated ER had a low specificity for its new ligand. This raises the question: "How was specificity for responses to different steroids regulated early in the evolution of steroid receptors?" Selective expression of these steroid-metabolizing enzymes provided specificity for different steroid responses in primitive vertebrates. 17β-hydroxysteroid dehydrogenase-type 1 (17β-HSD-type 1) and 17β-HSD-type 2, which preferentially catalyze the reduction and oxidation at C17 of androgens and estrogens, respectively, provide an example of this mechanism. Selective expression of either 17β-HSD-type 1 or 17β-HSD-type 2 can regulate synthesis or inactivation of androgens or estrogens in specific cells. Steroids also were important in the evolution of land animals, which began about 400 million years ago. Steroidogenic and steroid-inactivating enzymes were recruited to regulate steroid-mediated responses as organ function became more complex. For example, in the kidney 11β-HSD-type 2 prevents binding of glucocorticoids to the mineralocorticoid receptor, which is crucial for aldosterone-mediated regulation of electrolyte transport in the distal tubule. We propose that Δ5 steroids, such as dehydroepiandrosterone and its metabolites, were the ligands for the ancestral ER. Understanding the actions of Δ5 steroids in amphioxus and lamprey may shed light on adrenarche and neurosteroid actions in humans.

1. Steroid receptors, steroidogenic enzymes and steroid-inactivating enzymes regulate steroid hormone action.

The adrenal and sex steroids - aldosterone, cortisol, estradiol, progesterone and testosterone - regulate a wide range of physiological processes in vertebrates including reproduction, development and homeostasis. As a result, there has been much interest in understanding the mechanism of action of these hormones, and, in particular, how the receptors, which mediate their actions, regulate gene transcription. Although enzymes in steroidogenic tissues, such as adrenals, ovaries and testis, from where steroids were secreted into the circulatory system, were characterized, for the most part, steroidogenic and steroid-inactivating enzymes were not at the center stage in endocrine research. However, research on steroidogenic and steroid-inactivating enzymes increased, after it was realized that local expression of steroidogenic and steroid-inactivating enzymes has a key role in steroid hormone action (Funder et al., 1988; Edwards et al., 1996).

Enzymes that metabolize a single group, such as a hydroxyl or ketone, on steroid, can either activate or inactivate a steroid. This constitutes an economical on-off switch for regulating steroid hormone action because reversible inactivation and activation of steroids as they circulate through different organs conserves a complex molecule that requires over 25 steps for its synthesis. Selective expression in peripheral organs of enzymes that catalyze this on-off switch is an essential part of paracrine, autocrine an intracrine mechanisms for regulating steroid responses (Labrie et al., 2000), which together with the expression of steroid receptors in target tissues, provide a flexible mechanism for regulating diverse physiological responses vertebrates.

As will be discussed in this article, steroid receptors, steroidogenic enzymes and steroid-inactivating enzymes arose at about the same time in primitive vertebrates, and the subsequent co-evolution of these proteins was important in the evolution of complex developmental pathways found in vertebrates (Baker, 2003). Moreover, understanding the early events in the evolution of steroidogenic enzymes may shed light on adrenarche (Arlt et al. 2002; Rainey et al., 2002) and the actions of neurosteroids (Baulieu and Robel, 1998; Compagnone and Mellon, 1998) in humans.

2. Adrenal and sex steroid receptors are a vertebrate innovation.

Steroid receptors belong to the nuclear receptor family of transcription factors (Escriva et al., 2000). Nuclear receptors are found in vertebrates and invertebrates, but not in yeast or plants. Thus far, adrenal and sex steroid receptors have been found only in vertebrates (Baker, 2002a, 2003). This is consistent with PCR analysis of DNA from phylogenetically diverse animals (Escriva et al. 1997) and sequence analysis steroid receptors (Baker, 1997, 2001b, 2003; Escriva et al., 2000), both of which conclude that adrenal and sex steroid receptors arose at the beginning of the vertebrate line.

Recent work from lamprey, a jawless fish, and *Ciona intestinalis*, a urochordate, further narrows the timing of origins of adrenal and sex steroid receptors. Thornton (2001) found an ancestral estrogen receptor

(ER), progesterone receptor (PR) and corticoid receptor (CR) in the lamprey, a jawless fish. An analysis of the recently completed genome of *Ciona* indicates that this organism does not have receptors for adrenal and sex steroids (Dehal et al. 2002; Baker, 2003). Together these analyses suggest that adrenal and sex steroid receptors arose in a cephalochordate, such as amphioxus, or in a jawless fish [Figure 1].

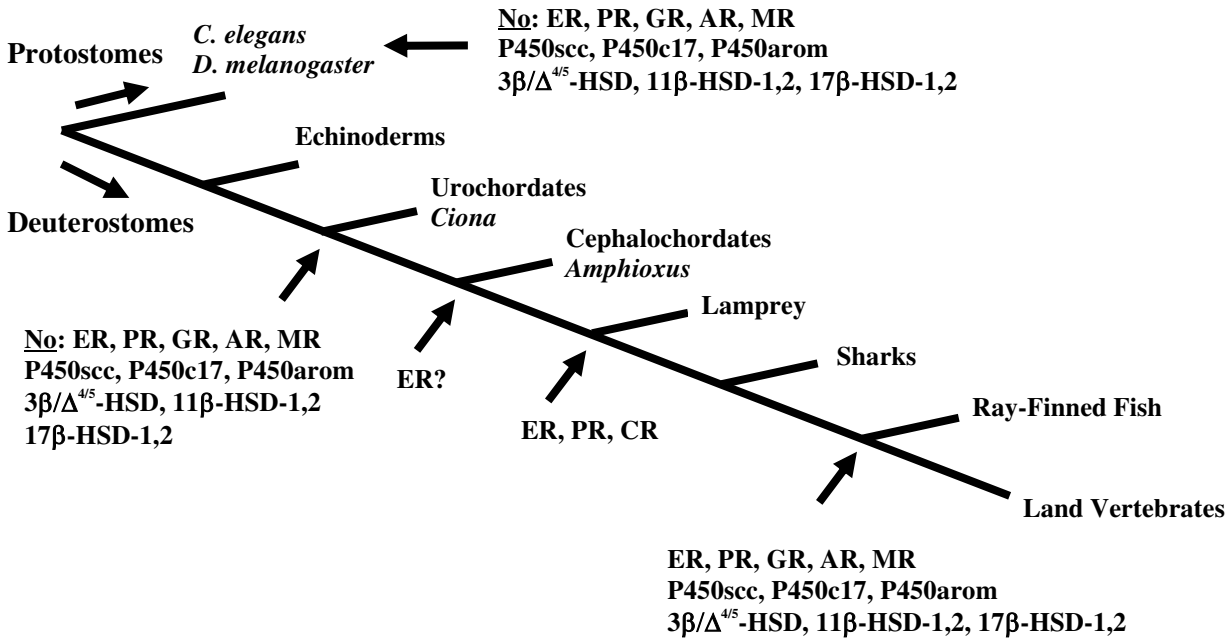

Figure 1. Adrenal and sex steroid signaling arose in a chordate.

Bony fish contain adrenal and sex steroid receptors and steroidogenic and steroid-inactivating enzymes. BLAST searches of the genome of *Ciona intestinalis* with these mammalian and fish sequences reveals that *Ciona* lacks genes coding for adrenal and sex steroid receptors and many of the key enzymes necessary for steroid synthesis (Dehal et al. 2002; Baker 2003). This suggests that the ancestral steroid receptor arose in a cephalochordate such as amphioxus (Baker, 1997, 2001b).

3. Several key steroidogenic and steroid-inactivating enzymes arose at the origin of vertebrates.

Figure 2 shows the cytochrome P450s and hydroxysteroid dehydrogenases that catalyze the synthesis of adrenal and sex steroid receptors from cholesterol (Nebert and Russell, 2002). Metabolism of cholesterol to pregnenolone by cytochrome P450scc is followed by conversion of pregnenolone to progesterone by $3\beta/\Delta^{5\text{-}4}$-hydroxysteroid dehydrogenase ($3\beta/\Delta^{5\text{-}4}$-HSD). This yields an active steroid, which also is a precursor for the other adrenal and sex steroids.

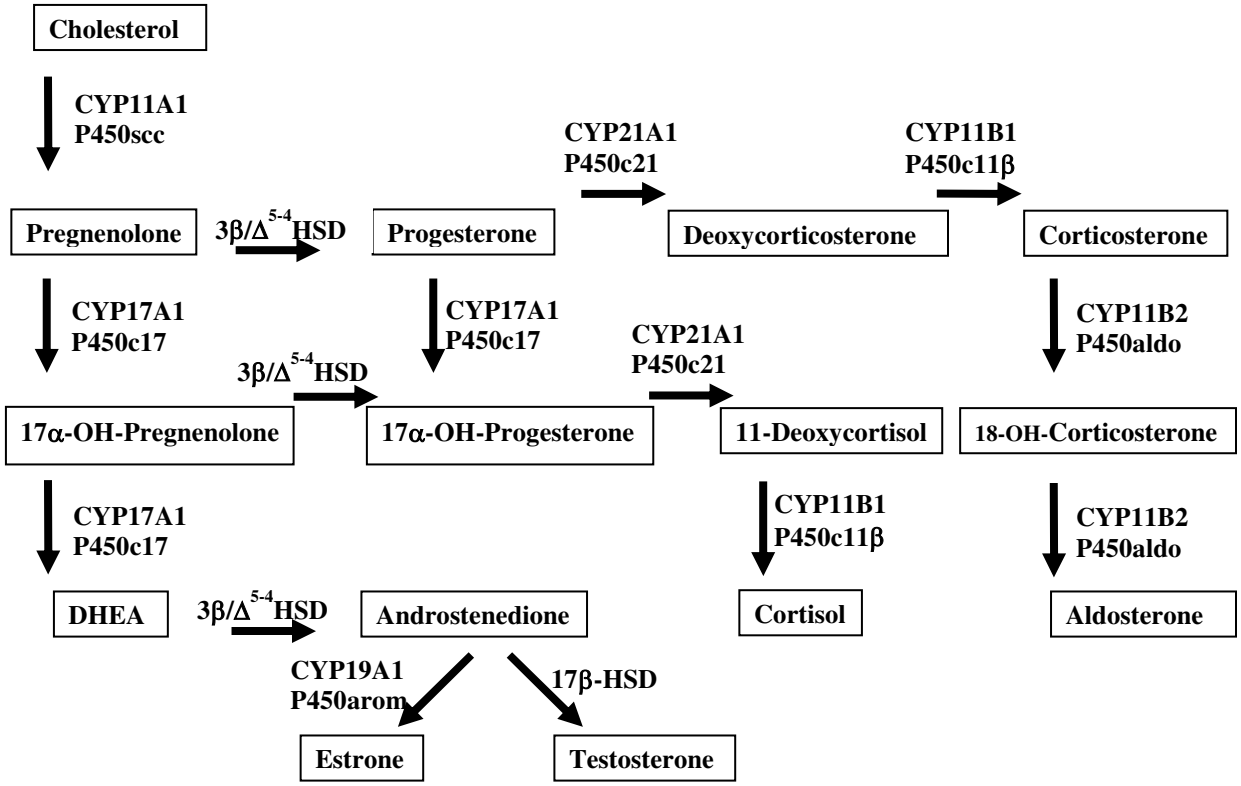

Figure 2. Enzymes involved in the synthesis of adrenal and sex steroids.

Progesterone is metabolized by various cytochrome P450s to corticosterone, cortisol and aldosterone [Figure 2]. Interestingly, androstenedione, a precursor of active androgens and estrogens, is formed dehydroepiandrostenedione (DHEA), a Δ5 steroid (Miller, 2002). Androstenedione is aromatized to estrone by cytochrome P450arom. 17β-hydroxysteroid dehydrogenase-type 1 (17β-HSD-type 1) and possibly other 17β-HSDs (Baker, 1996, 2001a; Peltoketo et al., 1999; Labrie et al., 2000) catalyze the formation of testosterone from androstenedione and estradiol from estrone. Thus, a combination of cytochrome P450s and steroid dehydrogenases catalyze the conversion of cholesterol to adrenal and sex steroids.

Other cytochrome P450s inactivate steroids by addition of hydroxyl groups, which facilitates steroid excretion and maintains proper steroid concentrations. Some steroid dehydrogenases inactivate steroids, as part of an on-off switch that is important in steroid homeostasis. Two important enzymes for this mechanism are 11β-hydroxysteroid dehydrogenase-type 2 (11β-HSD-type 2) and 17β-HSD-type 2. 11β-HSD-type 2 catalyzes the conversion of cortisol to cortisone, an inactive steroid. 17β-HSD-type 2 catalyzes the formation of estrone from estradiol and androstenedione from testosterone. Local expression of 11β-HSD-type 1 is important in the activation of cortisone and 11-dehydrocorticosterone to cortisol and corticosteorne,

respectively. Similarly, local expression of 17β-HSD-type 1 and other 17β-HSDs are important in the activation of androstenedione and estrone to testosterone and estradiol, respectively.

Cytochrome P450s and hydroxysteroid dehydrogenases that catalyze the synthesis and inactivation of adrenal and sex steroids are found in fish, which is consistent with the presence of adrenal and sex steroid receptors in fish (Escriva et al., 2000; Baker, 2003). Our searches of the genomes of the invertebrates, *Caenorhabidtus elegans* and *Drosophila melanogaster* with BLAST did not find evidence for cytochrome P450scc, cytochrome P450c17, cytochrome P450arom (M. Baker, unpublished) or 11β-HSD-type 1, 11β-HSD-type 2, 17β-HSD-type 1 and 17β-HSD-type 2 (Baker, 2001a). Moreover, a similar BLAST analysis of the *Ciona* genome reveals that it does not contain these dehydrogenases (Baker, 2003) or cytochrome P450s (Dehal et al., 2002, M. Baker, unpublished). Thus, key regulatory enzymes for steroid hormone action appear in chordates about at the same time as steroid receptors [Figure 1]. Together, the co-evolution of steroidogenic enzymes, steroid-inactivating enzymes and steroid receptors had an important role in the evolution of complex regulatory networks in vertebrates, contributing to vertebrate survival and diversification in the last 500 million years (Baker, 1996, 2003).

4. Evolution of adrenal and sex steroid receptors through a series of gene duplications.

Phylogenetic analysis of the adrenal and sex steroid receptors indicates that they arose by a series of gene duplications (Baker, 1997, 2001b, 2002b; Escriva et al., 1997, 2000; Thornton, 2001). Analysis of the evolution of the ligand-binding domain indicates that the ER is the most ancient of the adrenal and sex steroid receptors (Baker, 2001b, 2002b; Thornton 2001). A duplication of the ancestral ER led to the family of receptors that mediate the actions of 3-ketosteroids. The PR appears to be the first receptor of the 3-ketosteroid receptor family (Thornton, 2001). Subsequent gene duplications, followed by sequence divergence of the "extra" receptor, led to the other adrenal and sex steroid receptors: the androgen receptor (AR), the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) [Figure 3].

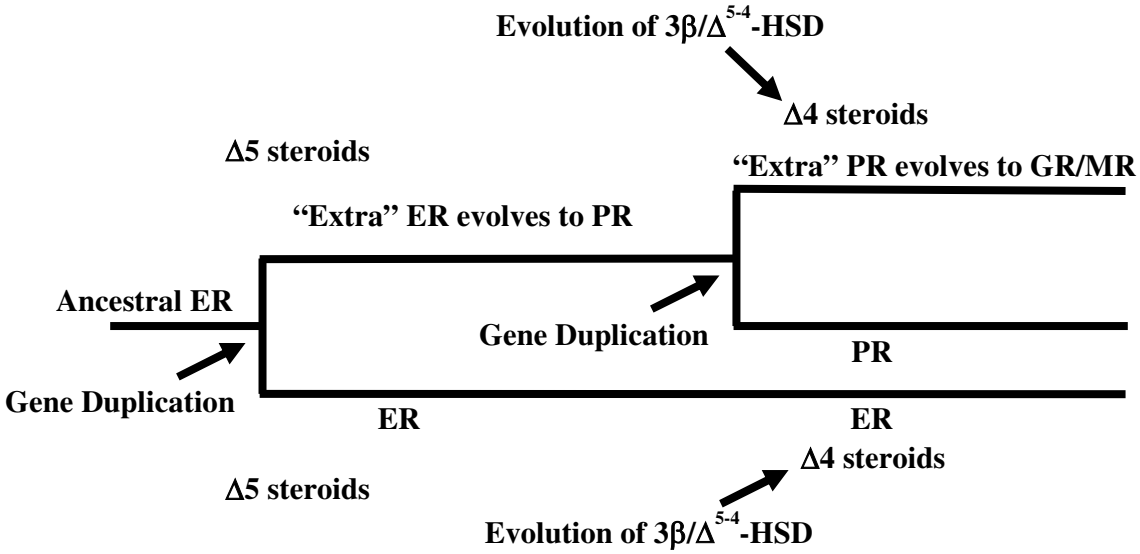

Figure 3. Evolution of adrenal and sex steroid receptors from an ancestral estrogen receptor.

Gene duplication of an ancestral estrogen receptor led to the 3-ketosteroid receptor clade. Initially, the ancestral ER had fuzzy recognition for Δ5 steroids. Thus, pregnenolone, 17α-hydroxypregenolone and DHEA may have bound the ancestral ER with sufficient affinity to act as either agonists or antagonists. The duplicated ER evolved into a nascent PR with greater specificity for the C17 side chain. During this interval, steroidogenic enzymes were important in regulating steroid hormone action in the ER and the nascent PR. The evolution of $3\beta/\Delta^{5-4}$-HSD added Δ4 steroids such as progesterone, cortisol, androstenedione, etc. as potential ligands for the ancestral ER, PR, AR, and GR, providing a selective force for the evolution of modern adrenal and sex steroid receptors.

5. Problem: Lack of steroid specificity early in the evolution of duplicated steroid receptors.

During the evolution of the duplicated ER to the PR, there was an interval when selectivity for progestins was low. Various mutations had to occur in the ligand-binding domain of the extra ER for the evolution of high affinity for a progestin, which compared to estradiol has a larger C17 side chain and an A ring with Δ4 unsaturated bond instead of the aromatic ring. During this interval, how was regulation of responses to a progestin accomplished?

Specificity for a response to estrogens or progestins could occur by each receptor binding to different DNA sequences. However, this is only a partial solution to specificity for different steroids because estrogens would still bind to the ligand-binding domain in the nascent PR. We propose that steroidogenic- and steroid-inactivating enzymes were part of the solution for achieving specificity in the ligand-binding domain that could bind a variety of steroids. Indeed, we propose this was a major mechanism for regulating the steroid response in primitive vertebrates; that is, steroidogenic- and steroid-inactivating enzymes were an integral part of the evolution of specificity for different physiological responses to adrenal and sex steroids.

For example, increased expression of $3\beta/\Delta^{5-4}$-HSD in target tissues would increase the local progestin concentration to a level that activates the nascent PR, before it evolved to have a high affinity for progestins. Alternatively or concomitantly, increased expression of 17β-HSD-type 2 would lower the local concentration of estradiol that competed with progestins for the evolving PR.

6. Fuzzy recognition of steroids by receptors in fish.

An insight into steroid hormone action 550 million years ago, when steroid receptors were evolving to respond to adrenal and sex steroids, comes from evidence that some steroid receptors in fish have less specificity for steroids than their mammalian homologs. The fish steroid receptor in which steroid specificity has been most thoroughly studied is eel PR, which binds 17α,20β-dihydroxyprogesterone, progesterone and 17α-hydroxyprogesterone with high affinity (Todo et al., 2000). The active progestin in fish is 17α,20β-dihydroxyprogesterone, which is not active in either in birds or mammals because the addition of a 17α-hydroxy group to progesterone reduces its affinity for these PRs to less than 1% of that of progesterone (Smith et al., 1974). Fish contain a 20β-hydroxysteroid dehydrogenase, which can increase the local concentration of the active progestin [Guan et al., 1999]. In mammals, expression of this enzyme will yield an inactive progestin, thus, quenching progestin action.

Similarly, eel AR (Todo et al., 1999; Ikeuchi et al., 2001) and Atlantic croaker AR (Sperry and Thomas, 1999) have diverse and different responses to androgens. Both eel ARα and ARβ are activated by 11-ketotestosterone, the main androgen in fish. However, eel ARs havesimilar transcriptional responses to 11-ketotestosterone, testosterone and 5α-dihydrotestosterone (5α-DHT) (Todo et al., 1999), in contrast to mammalian AR, which has a strong preference for 5α-DHT. Androstenedione at 100 nM will activate human AR, but not eel AR (Ikeuchi et al., 2001).

The two ARs in Atlantic croaker that have diverse steroid specificities, that contrast to the eel ARs. Atlantic croaker AR1 has highest affinity for testosterone, and over 10 fold lower affinity for 5α-DHT and almost 1000-fold lower affinity for 11-ketotestosterone (Sperry and Thomas, 1999). In contrast, Atlantic croaker AR2 has about the same affinity for testosterone and 5α-DHT and 5-fold lower affinity for 11-ketotestosterone. The basis for this diversity in binding and response to androgens in the different fish ARs is not understood.

7. Recruitment of steroidogenic and steroid-inactivating enzymes to regulate new physiological functions of steroids in land animals.

Vertebrate complexity increased with the colonization of land by amphibia and the subsequent radiations of reptiles, birds and mammals. This required the evolution of new organs and more complex

embryonic and postnatal development, in which steroids have an important role. During this time, there were important changes sequences and functions of steroid receptors and steroid-metabolizing enzymes as they co-evolved to regulate the increased complexity of land animals, such as placental mammals.

Mammalian steroid receptor specificity changed, as seen in the mammalian PR no longer responding to 17α-hydroxyprogestins. Consistent with the need for tighter control of steroid binding to receptors, is the slowdown in the rate of change of amino acid sequences of the steroid-binding domain in receptors in land animals compared to that in fish (Baker, 2001b).

Of important functional significance is the emergence of different steroids to regulate physiological responses in land animals. As mentioned earlier, 11-ketotestosterone is the active androgen in fish; 5α-DHT is the most active androgen in mammals. In fish, 11β-HSD catalyzes the conversion of 11-hydroxytestosterone to 11-ketotestosterone, the main androgen in males (Miura et al., 1991; Borg, 1994; Todo et al., 1999). In mammals, 11β-HSD does not have a significant role metabolizing androgens. Instead, the important enzyme is 5α-reductase, which converts testosterone to 5α-DHT (Wilson, 1999).

In land animals, 11β-HSD-type 2 has a key role in the mineralocorticoid response the kidney (Stewart and Krozowski, 1999). This coincides with the emergence of aldosterone as the main mineralocorticoid in land animals. Although aldosterone may be active in some fish, it appears that cortisol is the main mineralocorticoid in eel (Marsigliante et al., 2000), trout (Ducouret et al., 1995) and other teleost fish (Wendelaar Bonga, 1997). Cortisol binds and activates the fish MR, which also is called GR-1, to acknowledge its high affinity for glucocorticoids. The classical glucocorticoid receptor, GR-2, also is found in fish. Interestingly, trout MR binds 17α-hydroxyprogesterone, 11β-hydroxyprogesterone, 11-deoxycorticosterone, as well as, aldosterone, cortisol and corticosterone (Colombe et al., 2000). It is not known which of these other steroids are biologically important mediators of the mineralocorticoid response, and how specificity for one or more of these steroids is accomplished. Evidence from mammals suggests that enzymes that inactivate some of these steroids will be part of the regulatory mechanism.

Indeed, in land animals, the emergence of aldosterone as the main mineralocorticoid depends on its inertness towards metabolism by 11β-HSD type 2. This contrasts with the inactivation of cortisol to cortisone by 11β-HSD type 2. Thus, expression of 11β-HSD type 2 in the distal tubule of the kidney excludes cortisol from the MR and allows aldosterone to occupy the MR and regulate the mineralocorticoid response.

In placental mammals, 11β-HSD type 2 has been recruited to control the access of glucocorticoids to the GR in the fetus. Exposure of the fetus to glucocorticoids leads to hypertension in adults (Edwards et al., 1993, Seckl et al., 2000). Expression in the placenta of 11β-HSD type 2 prevents excess glucocorticoids from acting on the fetus.

Similarly, the levels of androgens and estrogens in the fetus are modulated by placental 17β-HSDs. However, regulation is more complex due to the presence in the placenta of 17β-HSD-types 1 and 7, which are reductases, and 17β-HSD-type 2, which is an oxidase (Peltoketo et al., 1999, Labrie et al., 2000; Baker, 2001a). The roles of these 17β-HSDs in controlling steroid access to the fetus are just beginning to be elucidated.

8. A paradox, the ER and not the PR is most ancient: What was the ancestral estrogen?

The finding that the ER is the most ancient of the adrenal and sex steroid receptors is paradoxical. The PR should be the most ancient receptor because progesterone is the first active Δ4 steroid, and progesterone is a precursor for other steroids including estradiol [Figure 2]. Indeed, there are several steps involved in the synthesis of estradiol from progesterone. Thus, the most parsimonious prediction is that the progesterone receptor was the first steroid receptor.

Thornton (2001) proposed a novel mechanism that explains how the first steroid receptor was the ER and responded to estrogens, despite the presence of progestins, glucocorticoids and androgens. He proposed that the upstream steroids, such as progesterone, cortisol, testosterone were inactive because a receptor with a ligand-binding domain for these steroids had not yet evolved. The presence of these upstream "orphan" ligands was a selective force for the evolution in duplicated receptors of a new steroid binding activity.

Alternatively, we proposed that a Δ5 steroid was the ligand for the ancestral ER (Baker, 2001b, 2002a). This is a more parsimonious model because the synthesis of DHEA, for example, from cholesterol requires one less enzyme, $3\beta/\Delta^{5\text{-}4}$-HSD, than the synthesis of androstenedione, which also must be metabolized further to form an estrogen [Figure 2]. Moreover, DHEA is the physiological precursor in the synthesis of androgens and estrogen via androstenedione in humans (Miller, 2002). Thus, the pathway for the synthesis of proposed ancient estrogens is still active in vertebrates. Based on our database searches (Baker, 2003) and those of Dehal et al. (2002), we propose that the complete cohort of enzymes required for the synthesis of Δ4 steroids, such as progesterone, glucocorticoids, and androgens, and for the conversion of androgens to estrogens had not yet evolved in a primitive chordate, containing the ancestral ER. Thus, a non-traditional steroid bound to the ER to regulate gene transcription in amphioxus and subsequently in jawless fish, such as hagfish and lamprey, in which the ER and other adrenal and sex steroid receptors first evolved. The notion that modern steroids were not the activators of ancestral steroid receptors over 525 million years ago is consistent with previously discussed evidence that the active androgens and progestins in fish differ from those in mammals. We do not require that this "non-traditional' estrogen have nM affinity for the ER, as is found in fish and land animals. Steroidogenic enzymes could synthesize sufficient steroid to occupy the ancestral ER. Indeed, high DHEA concentrations are found in primates during different stages in their life cycle (Cutler et al., 1978; Sklar et al., 1980; Arlt et al., 2002; Rainey et al, 2002).

Interestingly, there is "molecular fossil" evidence for estrogenic activity of Δ5 steroids in mammals. Δ5 androstene-3β,17β-diol (5-Adiol) has estrogenic activity in rats (Huggins et al., 1954) and binds to the ER in rat uterus (Garcia and Rochefort, 1979). Kuiper et al. (1997) reported that 5Adiol has nM affinity for recombinant ERα and ERβ. 5-Adiol is formed from DHEA by a 17β-HSD reductase [Figure 4].

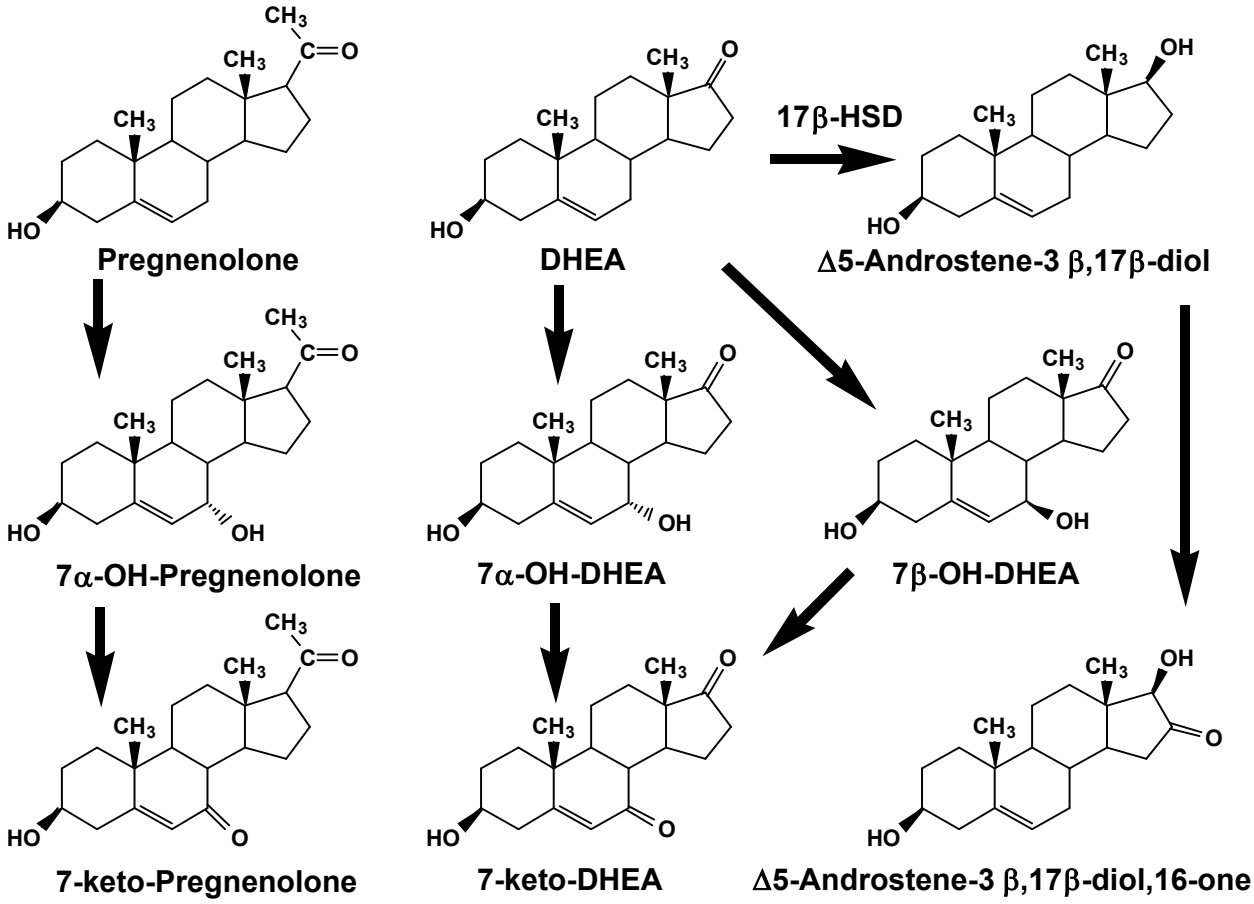

Figure 4. Δ5 steroids are potential ligands for the ancestral ER and PR.

Several Δ5 steroids derived from pregnenolone are active in humans and could activate the ancestral ER and PR. 5-Adiol (Kuiper et al., 1997) and 7-keto-DHEA and a 16-keto-derivative of 5-Adiol (Chang et al. (1999) activate the mammalian ER. DHEA, pregnenolone and 7-hydroxy-pregnenolone are neurosteroids (Akawa et al., 1992, Compagnone and Mellon, 1998; Baulieu and Robel, 1998). See Lathe (2002) for a more complete discussion of ancestral steroids.

Analogs of Δ5 steroids with substituents at C7 also are candidates as non-traditional steroids for the ancestral ER and PR, in the light of the evidence presented by Lathe (2002) that sterols with C7-hydroxyl groups were ancestral signals, predating the evolution of steroid hormone action. DHEA, pregnenolone and 5-Adiol are readily hydroxylated at C7 (Akwa et al., 1992; Lathe, 2002; Lardy, 2003). Moreover, these 7α-

hydroxy- and 7β-hydroxy-steroids can be oxidized to 7-oxo-steroids [Figure 4]. Support for the possibility that keto-Δ5 steroids were ligands for the ancestral ER comes from the report by Chang et al. (1999) that 100 nM 7-keto-DHEA and 16-keto derivative of 5-Adiol activated transcription mediated by the ER in MCF-7 breast cancer cells. These and other DHEA metabolites (e.g. 15-hydroxy- and 15-keto-DHEA, see below) may have higher affinity for ER in primitive chordates.

Steroids with C15 substituents also may be transcriptional activators of the ancestral ER and PR in view of the analysis of androgen and estrogen synthesis in male and female lamprey by Lowartz et al. (2003). Using a rigorous HPLC analysis of the metabolites of radioactive pregnenolone after incubation with lamprey ovaries or testes, Lowartz et al. found that the main products were C15-hydroxylated steroids, including DHEA, estradiol and testosterone, with no noticeable quantities of either estradiol or testosterone. Similarly, their analysis of the serum of male and female lampreys indicated the presence of 15-hydroxylated steroids, with little estradiol or testosterone. Lowartz et al. concluded that 15-hydroxylated forms of the "classical" steroids are the physiologically active androgens and estrogens in lamprey.

9. Clues for understanding adrenarche and neurosteroid action?

Adrenarche is the increased secretion by the adrenals of DHEA and its sulfate in children from age 6 to 8 years. Adrenarche is a unique and puzzling property of humans and chimpanzees (Cutler et al., 1978; Sklar et al., 1980; Arlt et al. 2002; Rainey et al., 2002). A better understanding of adrenarche may come from studies on the physiological responses mediated by Δ5 steroids in protochordates and primitive vertebrates.

Mammalian neurosteroids include pregnenolone, DHEA and their analogs (Corpechot et al., 1981; Akawa et al., 1992, Compagnone and Mellon, 1998; Baulieu and Robel, 1998; Lathe, 2002). Importantly, Compagnone and Mellon (1998) found that DHEA and its sulfate are important in the development of the rat brain. Our model suggests that modern neurosteroids were ligands for the ancestral ER and PR. Binding of these steroids to the ancestral ER would be consistent with the ancestral ER having an important role in the evolution of a more complex brain, which occurred in amphioxus and jawless fish (Baker, 1997, 2001b). In fact, the previously discussed estrogenic actions of 5-Adiol may be a relic of its earlier activity in the brain, suggesting that other Δ5 steroids derived from DHEA, pregnenolone or 17α-hydroxyprenenolone may be discovered that bind vertebrate adrenal and sex steroid receptors.

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