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Electrostatic interactions of androgens and progesterone derivatives with rainbow trout estrogen receptor

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Abstract

In primary cultures of immature male rainbow trout (rt) hepatocytes, vitellogenin (Vg) gene expression is regulated by E_2 via the estrogen receptor (ER). However, steroids other than estrogens can also stimulate Vg gene expression. These steroids are hardly converted into E_2 during incubation and their stimulatory activity is completely inhibited by tamoxifen implying rtER involvement. These steroids have no or a slightly positive charge on the Connolly surface. In contrast, steroids that failed to stimulate Vg gene expression had a strong positive or negative charge around rings C and D due to polarization. The amino acid sequences of the ligand binding domains (LBD) of rtER and human ERα have 57.7% homology; only one amino acid differs in the presumed steroid binding site. We modeled the three-dimensional structure of the LBD of rtER using X-ray crystallographic data for hERα in order to investigate the fit (structural and electrostatic) between steroid and rtER. Two factors are essential for binding to rtER: (i) hydroxyl or carbonyl groups near C3 and C17 of the steroids (hydrophilic regions) that can form hydrogen bonds with His(489), Arg(359), and Glu(318), (ii) a hydrophobic steroid nucleus that interacts with a hydrophobic region of the rtER LBD through van der Waals forces. If polar functional groups are present, the hydrophobic interaction between steroid and the rtER LBD is considerably weakened.

Keywords: Electrostatic interactions; Estrogen receptor; Vitellogenin gene expression; Rainbow trout

1. Introduction

Estradiol (E_2) is one of the most multifunctional steroids in species ranging from low vertebrates to mammals. It is involved in the up- and down-regulation of pituitary neuropeptides [1], the synthesis of hepatic proteins [2], growth hormone clearance [3], regulation of mammary gland growth and differentiation [4], and many metabolic functions [5], etc. Knowledge of the interaction of E_2 and other ligands with estrogen receptor (ER) is of importance for understanding of fundamental physiology and endocrine disruption. The structural features of ligands with estrogenic activity have been studied using receptor binding assays with human ERα(hERα) recombined into yeast. These studies have confirmed that steroids other than estrogens can bind to ER [6–8]. Recently, Brzozowski et al. [9] investigated the detailed tertiary structure of the ligand binding domain (LBD) of hERα by X-ray crystal analysis [9] although it is still unclear, from a structural standpoint, why a variety of steroids can bind to ER? At least one possible contributing factor to the broad range of ligands that can bind ER is that the cavity of the LBD is known from the crystallography structure to be almost twice the volume of estradiol. Thus, the volume of the LBD cavity may be a simple explanation for the variety of ligands that can bind ER.

A model for the study of estrogen action in many oviparous vertebrates (fish, amphibians, reptiles, birds) is the estrogen-induced synthesis of vitellogenin (Vg), a glycolipophosphoprotein precursor of egg yolk, by the liver during vitellogenesis. Vg is not specific to females. The males of most egg-laying vertebrates including Xenopus [10,11] and rainbow trout (rt) [12–14] also secrete large amounts of Vg into the circulation in response to estrogen. However, other classes of steroid hormone are also apparently able to induce Vg synthe-

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sis under certain conditions. According to one report on goldfish [15], massive doses of androgens produced Vg in vivo. In primary hepatocyte cultures from rt [16–20], androgens and progestagens induced Vg in some experiments [20] but not in others [19]. In our own studies on such cultures, Vg gene expression was induced by progesterone and by both low and high concentrations of androgens, but not by cortisol [21].

In the present study, we have compared the structural features of steroids that do and do not stimulate Vg gene expression and have investigated their possible interaction with a model of the LBD of rainbow trout estrogen receptor (rtER).

2. Materials and methods

2.1. Fish

Male immature rt (*Oncorhynchus mykiss*) (150–250 g) were kept in freshwater tanks at 15°C and fed once a day until collection of liver tissue.

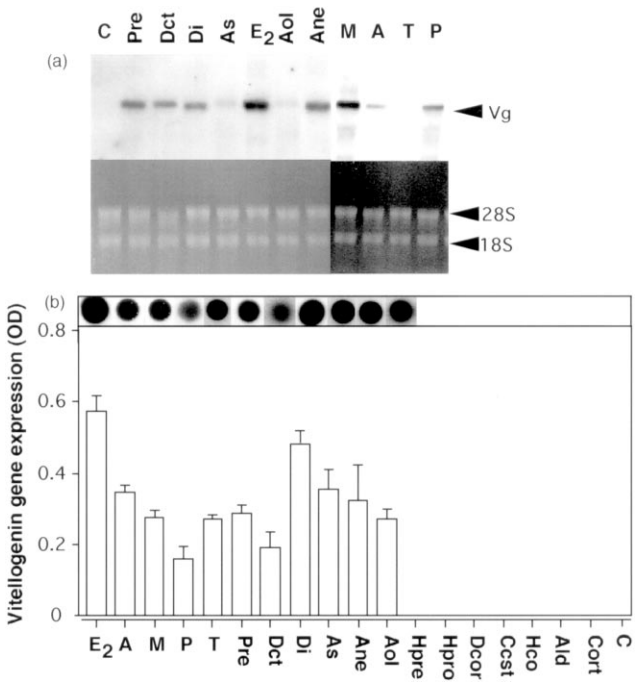

Fig. 1. Effect of various steroids on Vg gene expression in primary cultures of trout hepatocytes. (a) Northern blot analysis. Vg mRNA was detected in the total RNA (10 mg) by hybridization with a Vg probe. (C: control, E: E_2, P: progesterone, T: testosterone; A: androsterone, M: methyltestosterone, Aol: 5α-androstane-3α,17β-diol, Ane: 5α-androstane-3,17-dione, Pre: pregneno-lone, Dct: deoxycorticosterone, Di: dehydroisoandrosterone, As: 4-androstene-3,17-dione, Hpre: 17α-hydroxypregnenolone, Hpro: 17α-Hydroxyprogesterone, Dcor:11-deoxycortisol, Ccst: corticosterone, Hco: 18-hydroxycorticosterone, Ald: aldosterone, Cort: cortisol). (b) Dot blot hybridization of Vg mRNA (10 mg) extracted from hepatocyte cultures.

2.2. Hepatocyte cultures

Liver cells were dissociated as previously described [21] and plated in 60 mm plastic culture dishes (Falcon) at a density of 1×10^5 cells per dish. The cells were grown in Leibovits-15 medium (Gibco BRL) supplemented with 0.2 mM bovine insulin (Sigma), streptomycin (100 mg/ml), penicillin (70 mg/ml) and 5 mM Hepes (adjusted to pH 7.5). After 48 h of preculture, steroid dissolved in dimethylsulfoxide (DMSO) was added to the cells in culture. Eighteen steroids were chosen according to general metabolic path way of steroids [22], and tested at the high concentration of 2×10^{-5} M (17α-methyltestosterone, androsterone, 5α-androstane-3α, 17β-diol, 5α-androstane-3,17-dione, pregnenolone, progesterone, deoxycorticosterone, dehydroisoandrosterone, 4-androstene-3,17-dione, testosterone, estradiol-17β, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol, corticosterone, 18-hydroxycorticosterone, aldosterone, cortisol). Some experiments were performed in the additional presence of clomiphene or tamoxifen. Only DMSO (final concentration in the medium is 0.1%) was added to control cultures. After 24 h incubation at 15°C, the cells were harvested as previously described [21].

2.3. Northern blot analysis and vitellogenin mRNA quantification

Total RNA (10 mg) was extracted from the cultured hepatocytes incubated with steroid, fractionated by gel electrophoresis, transferred to Hybond-N+ membranes (Amersham) and hybridized with a digoxigenin-labeled Vg RNA probe. The DIG Luminescent Detection Kit (Boehringer Mannheim) was used to detect the hybridized probe. The membranes were then exposed to X-ray film. In the dot blot analyses, Vg mRNA was quantified by digital densitometry using the Bio Image system (Millipore). All steps in these procedures, the cloning and sequencing of Vg cDNA, and the preparation of sense and antisense RNA probes using this Vg cDNA have been previously described [21]. Chicken β-actin cDNA (kindly provided by Dr. Urano, University of Hokkaido, Japan) was used as a control.

2.4. Estradiol assay

Culture media were collected after 24 h incubation of hepatocytes with steroid and diluted 150 times with EIA buffer. E_2 content in the media was measured with an enzyme immunoassay kit (Cayman).

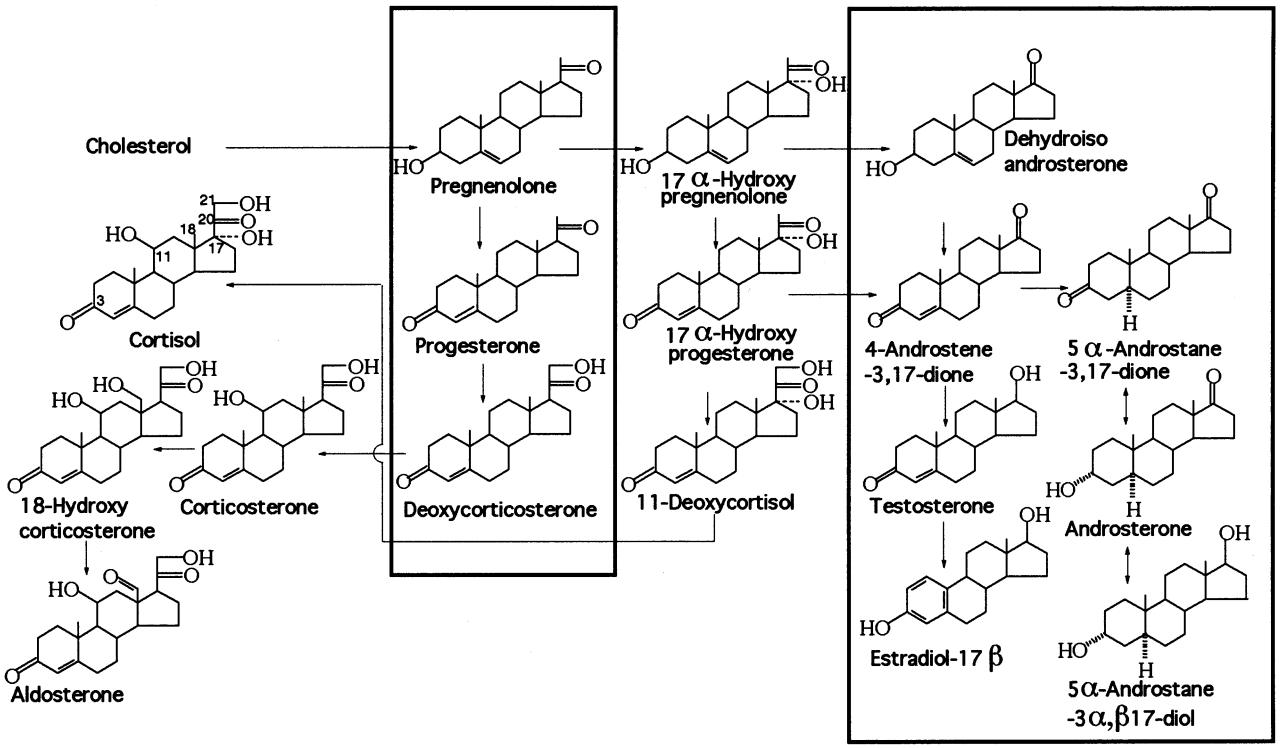

Fig. 2. Test steroids (those that stimulated Vg gene expression are boxed).

2.5. Modeling the ligand binding domain of rtER and analyzing the optimal fit with steroid

A model of the three-dimensional (3D) structure of the LBD of rtER was built from the X-ray structure of the E_2-hERα complex (Protein Data Bank accession code 1ERE) using the molecular modeling software QUATA/CHARMm (Molecular Simulation Inc., San Diego). Energy optimization and minimization of the initial structure were performed using restrained molecular dynamics [23] and QUATA/CHARMm.

3D steroid structures, based on their planar structures,were optimized with QUATA/CHARMm. The electrostatic potentials on the solvent accessible and Connolly surfaces were calculated, respectively, using QUATA/CHARMm and MOPAC 6.0, a semi-empirical molecular orbital calculation package. The optimized steroid structures were used to create rtER-steroid complexes on the basis of the published X-ray structure of hERα-17βE_2 [9] with QUATA/CHARMm.

2.6. Statistical analysis

Values for the optical density (OD: entire visible spectrum) of mRNA are expressed as mean ± SE. Statistical significance was determined by a one-way analysis of variance followed by a posteriori comparisons by Fisher's PLSD on the significant ANOVA results. Statistical significance was set at $P < 0.05$, and columns marked with the same letter do not show significant difference at the level.

3. Results

3.1. Stimulation of vitellogenin gene expression

There were two distinct groups of steroids, those that stimulated Vg gene expression, as visualized by a single band (6.6 kb) on Northern blot hybridization, and those that did not (Fig. 1). In the metabolic pathway illustrated in Fig. 2, stimulatory steroids are boxed in.

3.2. Inhibition of vitellogenin gene expression

Clomiphene and tamoxifen, although they can display some estrogenicity under other circumstances [24], did not stimulate Vg gene expression at concentrations ranging from 2×10^{-7} to 2×10^{-5} M. However, these concentrations of compound inhibited the Vg gene expression induced by 2×10^{-5}M E_2. In particular, tamoxifen was highly inhibitory (Fig. 3(a)). Concentrations of 2×10^{-5} and 2×10^{-4} M tamoxifen also inhibited Vg gene expression induced by other steroids (Fig. 3(b)). However, only the higher concentration significantly inhibited β-actin mRNA in the control experiment (Fig. 3(c)). Hardly any conversion of steroid

into E_2 was noted during incubation of any test steroid with culture medium for 24 h (Fig. 3(d)).

3.3. Steroid structures

In panel a of Fig. 4, the structures of the 11 steroids which stimulated Vg gene expression are superimposed. Panels b and c shows the electrostatic potential on the Connolly surface of these steroids (front and rear views, respectively). The 3D structures of these steroids are fairly similar. The electron-rich oxygens of the hydroxyl and carbonyl groups are positioned at C3, C17, C20 or C21 (Fig. 2). The distance separating the oxygens at each end of the steroids is between 10 and 12 Å whether there is double bonding (C4–C5, C5–C6), an aromatic A ring, or neither of these features within the steroid skeleton. Steroids that did not stimulate Vg gene expression had hydroxyl and/or carbonyl groups at C3, C11, C17 or C18. They all had a double bond at either C4–C5 or C6–C7. The electrostatic potential on their Connolly surfaces is shown in panels d–f.

There are clear differences in the electrostatic potentials of the stimulatory and non-stimulatory steroids. In the Vg-stimulating steroids including E_2, the Connolly surface is charged positively (in yellow in Fig. 4(b and c)) except around C3 and C17. In the non-stimulatory steroids, the hydroxy group at C17 creates a negatively charged zone around the C and D rings in three steroids with a common basic structure (17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol) (Fig. 4(d)). The other non-stimulatory steroids have a hydroxy group at C11 and C18 (corticosterone, 18-hydroxycorticosterone, aldosterone, cortisol) and display an area of strong positive charge around ring C (in red in Fig. 4(e, f)).

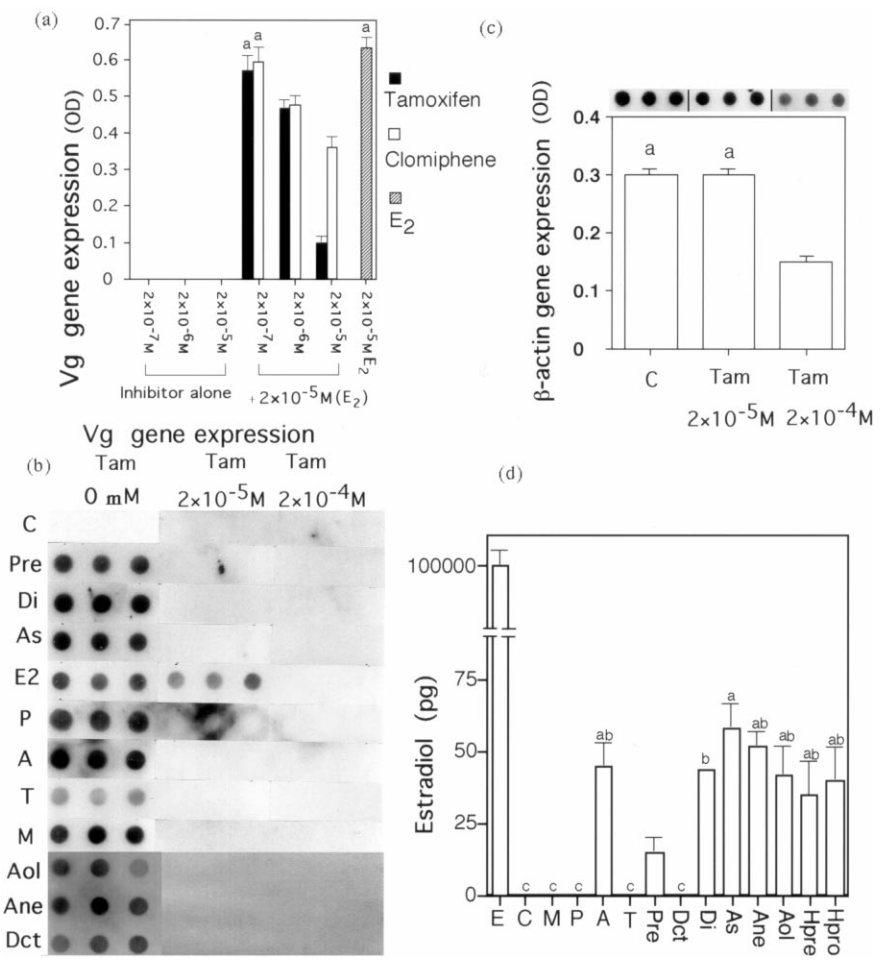

Fig. 3. (a) Effect of tamoxifen and clomiphene alone or in combination with 2×10^{-5} M E_2, and E_2 alone on Vg gene expression in rainbow trout hepatocytes (means of three fishes "*a*" = non-significant difference; $P > 0.05$). (b) Inhibition by tamoxifen of Vg expression induced by various steroids. Hepatocytes were cultured with test steroid alone or in the presence of tamoxifen (Tam). Vg mRNA extracted from total mRNA was detected by dot blot analysis. (For abbreviations, see legend to Fig. 1.) (c) Control experiment. β-actin mRNA was extracted from the total RNA (10 mg) in all control samples and all tamoxifen samples of panel (b). A representative dot blot is shown ("*a*" = non-significant difference; $P > 0.05$). (d) E_2 content in diluted culture media as measured by enzyme immunoassay after 24 h incubation("*a–c*" = non-significant difference; $P > 0.05$).

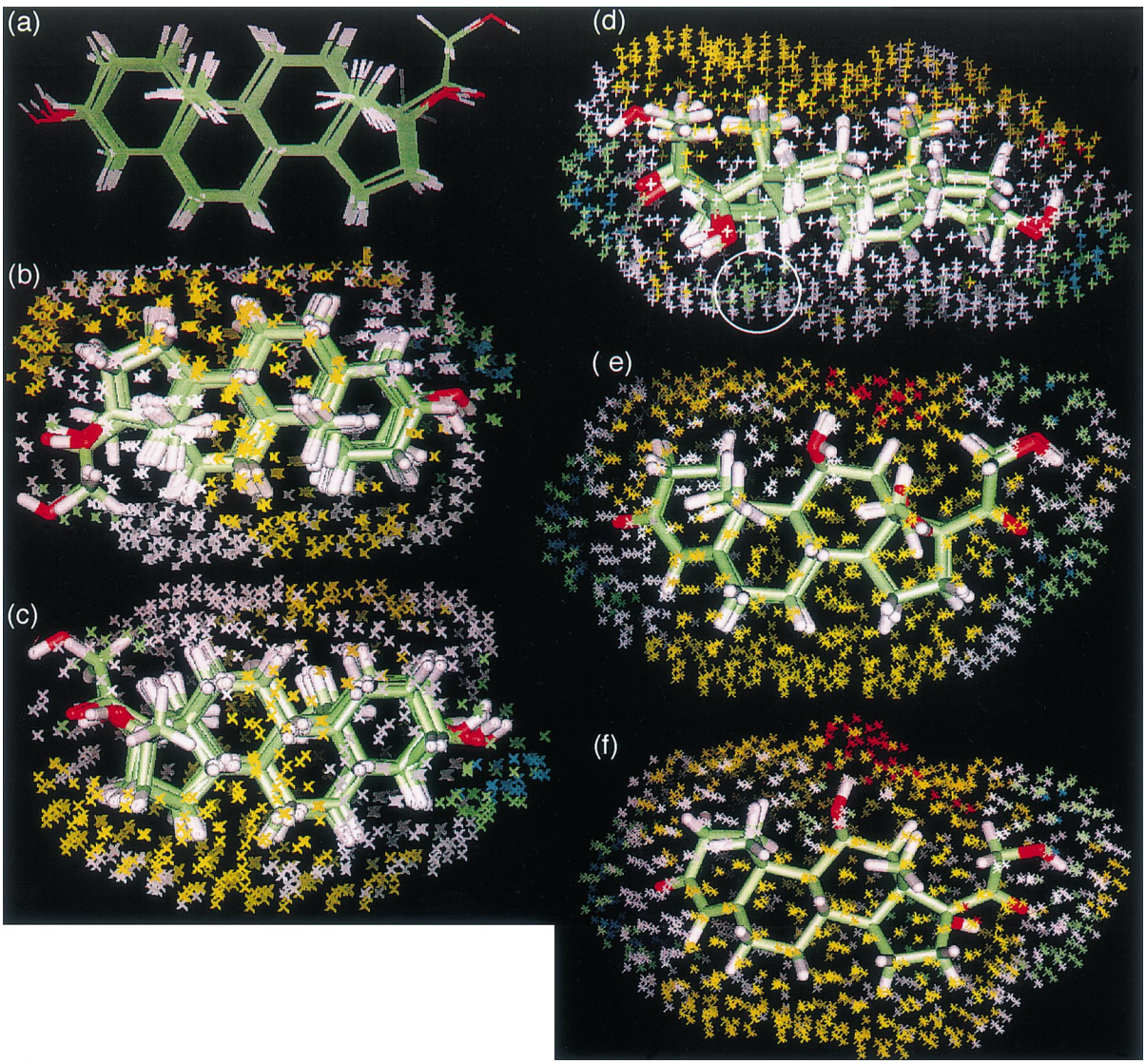

Fig. 4. Electrostatic potentials of steroids. (Energy color code: $-26.3 <$ blue < -16.3, $-16.3 <$ green < -6.9, $-6.9 <$ white < 3.1, $3.1 <$ yellow < 12.5, $12.5 <$ red < 22.6 kcal/mol. The values by MOPAC are converted in kcal/mol unit) Left-hand panels: (a) Superimposition of the steroids that stimulated Vg gene expression. Front (b) and rear (c) views of Vg stimulating steroids (17α-methyltestosterone, androsterone, 5α-androstane-3α,17β-diol, 5α-androstane-3,17-dione, pregnenolone, progesterone, deoxycorticosterone, dehydroisoandrosterone, 4-androstene-3,17-dione, testosterone, estradiol-17β). Right-hand panels: Electrostatic potentials of non-stimulatory steroids: (d) 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol (circle = polarity disturbing hydrophobic interaction); (e) Corticosterone, 18-hydroxycorticosterone, aldosterone; (f) cortisol.

3.4. Modeling of the ligand binding domains of rtER

The LBDs of rtER and hERα are aligned in Fig. 5; they have 57.7% homology. E_2 binds to18 amino acid residues in the LBD of hERα [9]. Of these, only Met (314) of rtER differs from the corresponding amino acid residue (Leu(349)) in hERα (Fig. 5). The root mean square distance between the atoms of the two LBDs is 0.87Å, suggesting that these LBDs are similar (Fig. 6(a)). The details of the binding pocket in each LBD are shown in Fig. 6(b). The nature and relative positions of the amino acid residues interacting with E_2 are almost the same. In both LBDs, E_2 would form hydrogen bonds with His(489) and Glu(318) (Fig. 7(a)).

Panel c of Fig. 6 shows a typical steroid (deoxycorticosterone) that stimulates Vg gene expression, panel e shows a steroid (11α-hydroxypregnenolone) that does not. The corresponding solvent accessible surfaces are given in panels d and f, respectively, and indicate that the hydroxy group at C17 of 11α-hydroxypregnenolone exists negative electrostatic potential zone (in blue) in the vicinity of rings C and D. We docked deoxycorticosterone into the modeled rtER binding pocket (panel g). The hydroxy group at C21 could form a hydrogen bond with Leu(490), the carbonyl group at C20 with

His(489) and the carbonyl group at C3 with Arg(359). The steroid skeleton could interact with hydrophobic amino acids such as Met(308), Leu(311), Phe(369), Leu(367), Met(386), Ile(389), Leu(393), Phe(390), Gly(486), Leu(349), Met(353), Leu(352), and Ala(315), thus reinforcing the stability of overall binding to rtER. All the steroids that stimulated Vg gene expression fitted this general binding pattern, i.e. formation of a hydrogen bond with His(489) at one extremity and with Arg (359) (carbonyl group) or Glu(318) (hydroxy group) at the other (Fig. 7(b)).

The electrostatic potential of the hydrophobic binding pocket of the LBD of modeled rtER is shown in Fig. 6(h). The pocket is weakly charged (green to yellow, i.e. from about -5.2 to $+16.7$ kcal/mol) except around His (489), Leu(490), Glu(318) and Arg(359). These regions would thus be in contact with low polarity residues; this corresponds well with the electrostatic potential data for the steroids.

4. Discussion

Our study was in two parts. First an investigation of the ability of a variety of steroids to stimulate Vg gene expression in rt hepatocytes in culture, then an analysis of the possible interactions of these steroids with a model of the 3D structure of the LBD of rtER based on a published X-ray crystal structure for the E_2-LBD-hERα complex [9].

The test-steroids fell into two distinct classes, those that did and those that did not stimulate Vg gene expression. Since the active steroid concentration was relatively high (2×10^{-5}M) for steroid classes (androgens and progestagens) other than estrogens, we checked whether their might not be conversion into E_2 by enzyme immunoassay. Hardly any such conversion was noted. Both active steroids and an inactive steroid such as 17α-hydroxypregnenolone gave rise to a similar, very low E_2 level (< 40 pg E_2) (Fig. 3(d)). The E_2 level was equally low for 17α-methyltestosterone, a non-aromatizable androgen which stimulates Vg gene expression (data not shown). However, because all the test-steroids except for 17α-methytestosterone are part of a general steroid metabolic pathway, there was a possibility that they might be converted into each other during incubation. HPLC studies have shown that testosterone is metabolized in rainbow trout hepatocyte cultures primarily into testosterone-glucuronide and androstenedione but also into the minor metabolites 6β-hydroxytestosterone, 16α-hydroxytestosterone, and 16β-hydroxytestosterone [25].

Stimulation of Vg gene expression by high concentrations of androgens and progestagens seeks the question whether these compounds interact directly with rtER. In our experiments, this stimulation was inhibited by the estrogen antagonist, tamoxifen. Although this suggests that ER might be somehow involved in the stimulation mechanism, tamoxifen is known to be able to act via other signaling pathways involving, for instance,

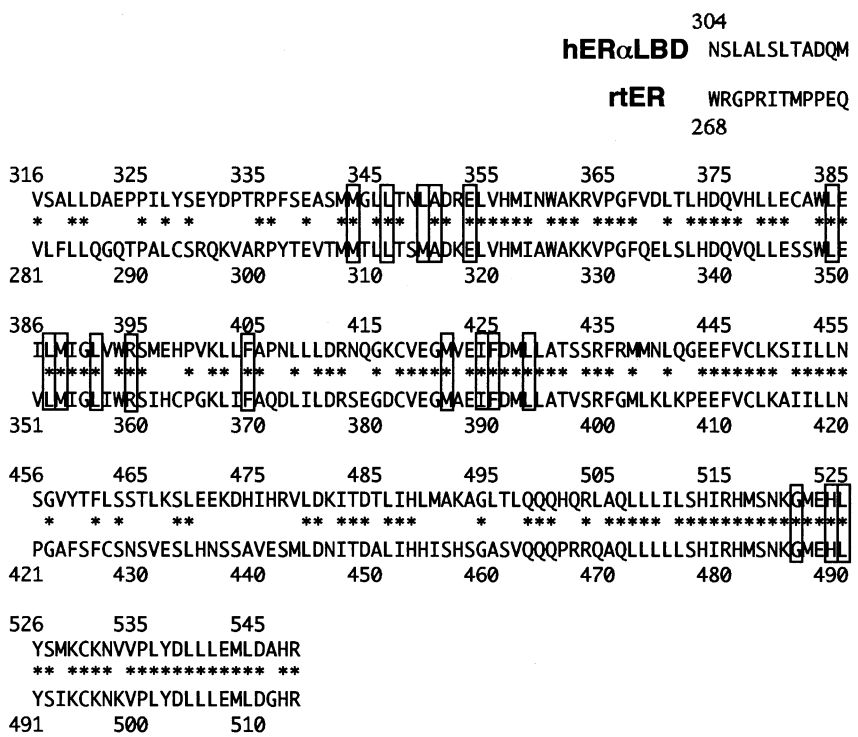

Fig. 5. (a) Sequence alignment of the LBDs of hERα and rtER (* = 3D identical residue; boxing = 3D binding site residues).

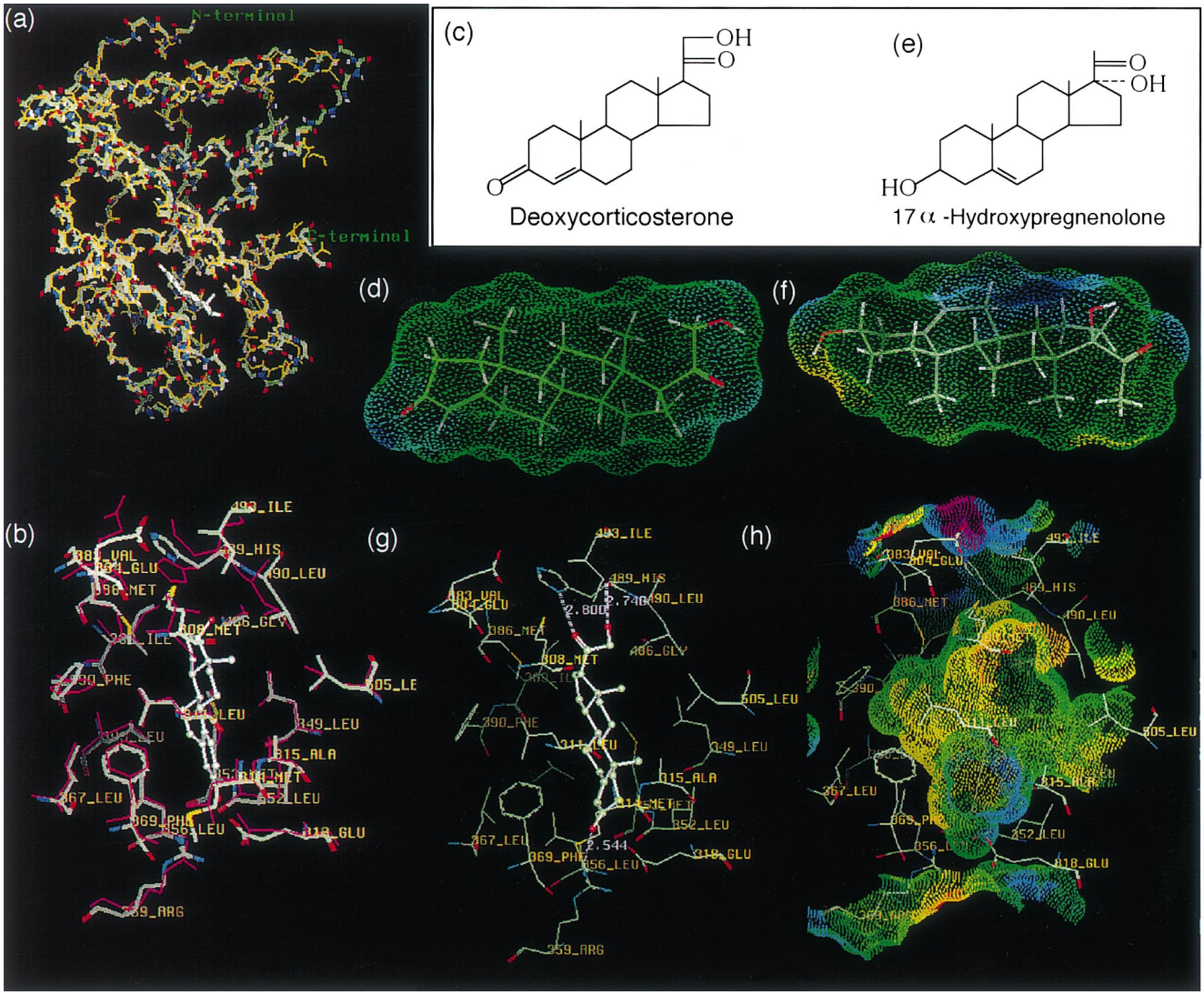

Fig. 6. Structures of the LBDs of hERα and rtER. (a) hERα (yellow) and rtER (green). (b) Binding site of hERα (red) and rtER (green) with bound E_2. (c, e) Chemical structures of deoxycorticosterone and 17α-hydroxypregneno-lone; (d, f) Electrostatic potentials of deoxycortico-sterone and 17α-hydroxypregnenolone. (g) Binding site of rtER with bound deoxycorticosterone. (h) Electrostatic potential of rtER (deep purple: −27.2 to −19.9, dark blue: −19.9 to −12.6, light blue: −12 to −5.2, green: −5.2 to 2.1, olive green: 2.1 to 9.4, yellow: 9.4 to 16.7, orange: 16.7 to 24.0, red: 24.0 to 31.4 kcal/mol).

protein kinase C [26]. Tamoxifen inhibits calcium/calmodulin-dependent CAMP phosphodiesterase and reduces calcium currents [27]. In all events, our experiments with an inhibitory concentration of tamoxifen showed no signs of cell toxicity.

The structural requirements for steroid binding to various classes of receptor have been studied by many teams. Comprehensive reviews have been published [28]. Highly novel compounds have been used to predict interactions between ligand features, such as the aromatic ring of estrogens, and LBD amino-acid residues [29]. The recent X-ray crystallographic analysis of the LBD of hER [9] has revealed that the 3-hydroxy group of E_2 forms a hydrogen bond with Glu(353), whose equivalent is not possible in hPR [30], and that the 17-hydroxyl binds to His(524). Moreover, a strong hydrophobic interaction occurs between the steroid skeleton and ER. So far, however, published data have not yet provided an explanation for the fact that, depending upon circumstance, a relatively wide variety of compounds seem to be able to bind to hERαLBD.

Part of the problem resides in the difficulty in judging what is a significant and meaningful binding affinity for ER and in the multifactorial nature of ligand binding assays which can be analyzed in vitro and in vivo independent of ER-DNA binding such as interaction of the E_2–ER complex with the estrogen response element responsible for induction of transcription [31].

Recently, the quantitative structure-activity relationships (QSAR) between estrogens and ER have been modeled [32]. We used a similar approach but focused on the structure of steroids that stimulate Vg gene

expression, which is generally considered to be an estrogenic response but which, as we have seen above, can also occur with other classes of steroid. We noted that there was good concordance between the electrostatic potentials of the active steroids, whether estrogens, androgens or progestagens, and the electrostatic potential of the modeled rtER binding pocket. On the other hand, inactive steroids would seem to lead to unstable interactions. If 17α-hydroxypregnenolone were to bind like E_2, its interactions with Met (386) or Phe (390) would be unstable as would be the interactions of corticosterone with Leu (352) or Leu (349).

All the steroids that stimulated Vg gene expression would form a hydrogen bond with His (489) of rtER at one extremity and with Arg (359) or Glu (318) at the other. Both 17-hydroxyl and 17-keto steroids would interact with the imidazole-ring of His (489), rotating the side-chain or exchanging protons with the N of the ring ($pK_a = 6.0$). The carbonyl group at C3 of, for instance, deoxycorticosterone would bind to the right-hand side of the amido NH_2 group of Arg (359). However, this could not occur with for testosterone, which is a smaller molecule than deoxycorticosterone. The hydrogen bond would become too weak. We therefore predict that the amido NH_2 group of Arg(359) will shift slightly to form a hydrogen bond with Glu (318), leaving the left-hand side of the amido NH_2 group of Arg (359) to form a hydrogen bond with testosterone (Fig. 7(b)). The role of Glu (318) is supported by the results of a point mutation study in which Glu (358) – the counterpart of Glu (318) (Fig. 5) – was found to play a significant role in discriminating between estrogens and androgens [33]. Unfortunately, although two other point mutations were investigated, Arg (394) – the counterpart of Arg (359) – was not one of them. Water molecules would correctly orient and position the discriminating glutamate or glutamine residue in hER [9] and hPR [30]. We did not depict them because they are not always present in crystals. They are not an absolute requirement to explain ligand binding.

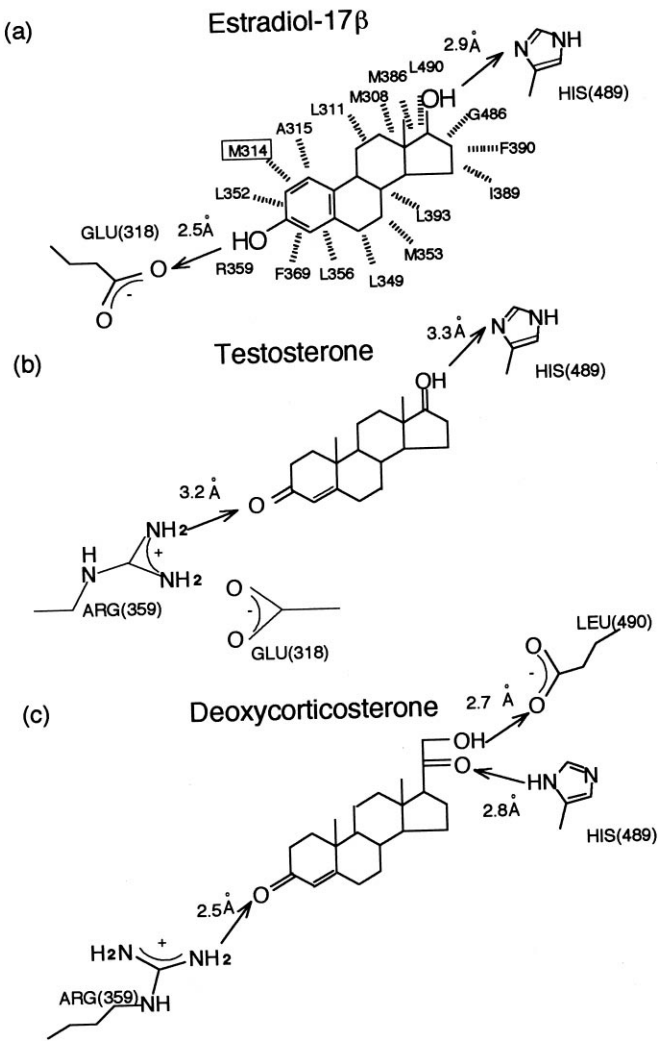

Fig. 7. (a) Amino acid residues to which E_2 might bind in rtER (identical residues to those in hERα except for M(314)). (b) Possible hydrogen bonds between deoxycorticosterone and rtER. (c) Possible hydrogen bonds between testosterone and rtER.

In conclusion, we have provided evidence for a plausible interaction between steroids stimulating Vg gene expression and a model of the LBD of rtER. This type of interaction might explain the stimulatory action of some androgens and progestagens in rainbow trout hepatocytes in culture.

Acknowledgements

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