Specific hydroxylations determine selective corticosteroid recognition by human glucocorticoid and mineralocorticoid receptors

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Abstract The ligand binding domains of the human mineralocorticoid receptor (hMR) and glucocorticoid receptor (hGR) display a high sequence homology. Aldosterone and cortisol, the major mineralocorticoid and glucocorticoid hormones, are very closely related, leading to the cross-binding of these hormones to both receptors. The present study reports on the mechanism by which hMR and hGR are activated preferentially by their cognate hormones. We found that the ability of corticosteroids to stimulate the receptor's transactivation function is depending on the stability of the steroid-receptor complexes. In the light of a hMR structural model we propose that contacts through the corticosteroid C21 hydroxyl group are sufficient to stabilize hMR but not hGR and that additional contacts through the C11- and C17-hydroxyl groups are required for hGR.

Key words: Aldosterone; Cortisol; Mineralocorticoid; Glucocorticoid; Steroid receptor

1. Introduction

The major human mineralocorticoid and glucocorticoid hormones, aldosterone and cortisol, are structurally very similar. They act by binding to their cognate receptors, the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) [1]. MR and GR belong to a large family of ligand-activated transcription factors that are structurally and functionally related [2,3]. They are characterized by a conserved DNA binding domain and a C-terminal ligand binding domain (LBD) essential for chaperone protein interaction, dimerization and hormone-dependent transactivation.

The way in which aldosterone acts through its own receptor has been a puzzle for some time for two main reasons. Aldosterone and cortisol both bind to the human MR (hMR) with the same affinity, and the plasma glucocorticoid concentration is 100–1000-fold higher than that of aldosterone. Consequently, most of the hMR should be occupied by glucocorticoids, resulting in a sodium retention. The role of the 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) in mineralocorticoid/glucocorticoid selectivity has been established during the past decade [4,5]. This enzyme metabolizes 11β-hydroxylated glucocorticoids, but not aldosterone, into 11-keto derivatives with a low affinity for the MR [6]. Receptor-associated mechanisms might also contribute to aldosterone selectivity, especially in aldosterone-sensitive tissues where 11βHSD2 and MR are not coexpressed [7].

Studies of a hMR-LBD homology model and mutagenesis analysis have identified several residues involved in the interaction with the functional groups common to aldosterone and cortisol [8]. The Gln-776 and Arg-817 residues interact with the 3-keto group and Asn-770 with the 21 hydroxyl function. Aldosterone and cortisol differ at positions C11, C17 and C18. Aldosterone has a 11–18 hemiketal bridge and cortisol has C11β and C17α hydroxyl groups. Despite these differences, aldosterone and cortisol display similar affinity for MR. Nevertheless MR is more sensitive to aldosterone than to cortisol [9–12], an observation that is not 11βHSD-dependent [12]. Conversely, cortisol better stimulates the GR transactivation than aldosterone, although these two hormones bind to the hGR with an affinity of the same order of magnitude [9,11].

This study was therefore carried out to explore the mechanism of hormonal recognition by each class of corticosteroid receptors. The stability of hMR and hGR associated with aldosterone, cortisol or dexamethasone, a synthetic glucocorticoid, was examined together with the ligand-induced hMR and hGR conformation changes. The receptors transactivation activity in response to corticosteroids bearing hydroxyl groups at C11, C17 and/or C18 position was measured by cotransfection assays. The ability of hMR and hGR to be selectively activated by their cognate hormones is due to specific steroid receptor contacts that stabilize the active receptor conformation.

2. Materials and methods

2.1. Chemicals

[1,2-^{3}H]Aldosterone (40–60 Ci/mmol), [1,2,6,7-^{3}H]cortisol (63 Ci/mmol), [1,2,4-^{3}H]dexamethasone (42 Ci/mmol) and ^{35}S-methionine were purchased from Amersham (Les Ulis, France). Non-radioactive aldosterone (Aldo), corticosterone (B), 11-deoxycorticosterone (DOC), cortisol (F), 11-deoxycortisol (cortexolone, S), dexamethasone (Dex), chymotrypsin and trypsin were obtained from Sigma (St. Louis, MO, USA). 18-hydroxycortisol (18OHF) and 18-oxocortisol (18oxoF) were synthesized as previously described [13,14]. The structure of the steroids used are shown in Fig. 1. Entensify Universal Autoradiography Enhancer was obtained from Du Pont-New England Nuclear (Boston, MA, USA). Products for cell culture were

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from Gibco-BRL (Cergy Pontoise, France). The profection mammalian transfection system and the TNT T7-Quick coupled transcription/translation system were obtained from Promega (Charbonnières, France). Steroid solutions were prepared in ethanol, dried and suspended in 50% (v/v) polyethylene glycol 300 prepared in TEG (20 mM Tris–HCl, 1 mM EDTA and 10% glycerol, pH 7.4 at 20°C) to give a 5% (v/v) final concentration of polyethylene glycol 300 in the lysate.

2.2. Construction of expression plasmids

The plasmid pchGR was constructed by excising a *Kpn*I–*Xho*I fragment including the entire hGRα coding sequence and about 110 bp and 500 bp of the 5′ and 3′ untranslated regions from the plasmid pRShGRα [15] and inserting it into pcDNA3 (Invitrogen NV, Leek, The Netherlands).

2.3. Cell culture and transfection

COS-7 cells were cultured in six-well trays in the presence of Dulbecco's minimal essential medium (DMEM, Gibco-BRL, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2. Cells were maintained in the medium supplemented with 10% charcoal-stripped FCS 4 h before and throughout the transfection procedure. Cells were transfected by the phosphate calcium precipitation method (Promega system). The phosphate solution, prepared for six-well trays, contains 5 μg of one of the receptor expression vectors pchMR [8] or pchGR, 10 μg of pFC31Luc that contains the MMTV promoter driving the luciferase gene [16] and 5 μg of pSVβ including the gene encoding β-galactosidase. The steroids to be tested were added to the cells 12 h after transfection and incubation continued for 24 h. Cell extracts were then prepared and assayed for luciferase [17] and β-galactosidase activities [18]. Transfection efficiency was standardized by dividing the relative light units obtained in the luciferase assay by the optical density obtained in the β-galactosidase assay.

2.4. Steroid binding at equilibrium

The hMR or hGR were expressed in vitro using the T7-coupled rabbit reticulocyte lysate system and the lysate was diluted two-fold with ice-cold TEGWD buffer (20 mM sodium tungstate and 1 mM dithiothreitol in TEG). It was then incubated for 4 h at 4°C with various concentrations of [^{3}H]steroid (0.1 to 100 nM). Bound (B) and free (F) steroids were separated using dextran-charcoal as previously described [8]. The change in B as a function of F was analyzed [19] and the dissociation constant at equilibrium, K_d, was calculated.

2.5. Kinetic experiments

The hMR or hGR was translated and the lysate was diluted two-fold with ice-cold TEGWD buffer and then incubated with 10 nM [^{3}H]steroid for 1 h at 20°C. One half of the labelled lysate was kept at 20°C to measure the stability of the [^{3}H]steroid–MR complexes, and the other was incubated with 10 μM of unlabeled steroid for various periods. Bound and free steroids were separated with dextran-charcoal. Parallel incubations containing [^{3}H]steroid plus a 1000-fold excess of unlabeled steroids were used to calculate the non-specific binding. The half-lives of the steroid-receptor complexes ($t_{1/2}$) were calculated from the equation $B_{(t)} = B_{(0)}\ e^{-(k_{-1}t)}$, where $B_{(0)}$ and $B_{(t)}$ are the specific steroid binding at times 0 and t of the dissociation period. $B_{(t)}$ is corrected for the stability of steroid binding at each dissociation time.

2.6. Limited proteolytic digestion of translated receptors

[^{35}S]Receptor synthesized in vitro were incubated with or without unlabeled steroid (10^{-10} to 10^{-5} M) for 10 min at 20°C. Chymotrypsin (100 μg/ml) was added to [^{35}S]-hMR and trypsin (60 μg/ml) to [^{35}S]-hGR. Aliquots of digestion products (1 μl) were mixed with 20 μl protein loading buffer, boiled for 5 min, loaded onto a 12.5% SDS-polyacrylamide gel and electrophoresed. The gels were fixed for 30 min in methanol/acetic acid/distilled water (30:10:60), treated with Entensify, dried and autoradiographed at -80°C overnight. Autoradiographs were scanned by image analysis (Optilab, Graftek, France). Results are given as optical density, expressed in arbitrary units.

3. Results

3.1. Corticosteroids differently protect hMR and hGR against proteolysis

It has been shown that limited chymotrypsin digestion of hMR generates a major 30 kDa fragment whose resistance to chymotrypsin is increased by aldosterone binding, indicating a change in the receptor compaction [20,21]. To examine the influence of the steroid on the 30 kDa fragment resistance, hMR was incubated with aldosterone, cortisol or dexamethasone (10^{-10}–10^{-5} M) and then treated with 100 μg/ml chymotrypsin for 10 min at 20°C. Under these conditions the ligand-free hMR was completely digested by chymotrypsin (data not shown), but incubation with steroids increased the fraction of undigested 30 kDa fragment in a dose-dependent manner (Fig. 2). Aldosterone protected the hMR 30 kDa fragment against proteolysis better than did glucocorticoids: 8×10^{-9} M aldosterone and 5×10^{-8} M of cortisol were required to recover 50% of the undigested fragment, whereas 10^{-5} M dexamethasone did not fully protect the hMR. Digestion of hGR with trypsin generates 27 and 30 kDa fragments whose respective abundance depends upon the incubation time and trypsin concentration [22,23]. Incubation of the ligand-free hGR with 60 μg/ml trypsin for 10 min at 20°C led to a complete digestion (data not shown). Incubation of hGR with steroids before trypsin treatment led to a 27 kDa fragment whose intensity is depending upon the steroid concentration: 5×10^{-9} M dexamethasone and 8×10^{-7} M cortisol were required to recover 50% of the undigested fragment, whereas 10^{-5} M aldosterone did not fully protect the hGR, indicating

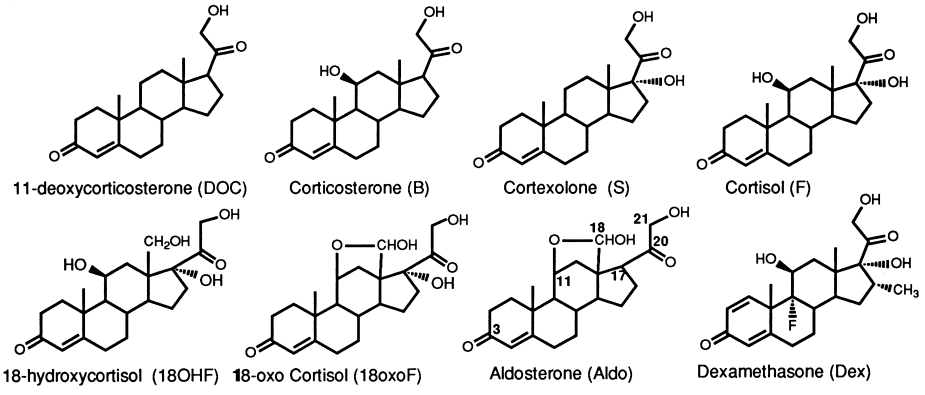

Fig. 1. Natural and synthetic corticosteroids.

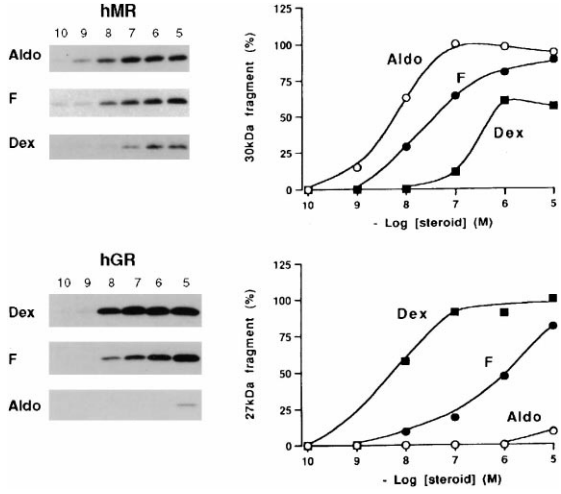

Fig. 2. Effect of corticosteroids on hMR and hGR proteolysis. ^{35}S-labeled hMR or hGR were produced by translation in vitro and incubated with of unlabeled steroid (10^{-10}–10^{-5} M). Steroid-hMR complexes were treated with 100 μg/ml chymotrypsin and steroid-hGR complexes with 60 μg/ml trypsin for 30 min at 20°C. The digestion products were analyzed by electrophoresis in a 12.5% (w/v) polyacrylamide gel and autoradiographed. The bands corresponding to the hMR 30 kDa fragment and hGR 27 kDa fragment were scanned and quantified. The intensities of the proteolysis fragments recovered from each receptor are expressed as a function of steroid concentrations, taking the maximum intensity recovered for each receptor as 100%.

a low efficiency of aldosterone in protecting hGR against proteolysis (Fig. 2).

3.2. hMR and hGR are differently stabilized by corticosteroids

The dissociation constants at equilibrium (K_d) of steroids for in vitro expressed hMR or hGR were measured from Scatchard plots and the half-life times of steroid-receptor complexes were calculated from dissociation kinetics studies. Aldosterone, cortisol and dexamethasone have similar affinity for the hMR (K_d: 0.5–0.8 nM, Table 1). Aldosterone dissociated much more slowly from the hMR than did cortisol or dexamethasone, indicating that the aldosterone-hMR complexes were more stable than the glucocorticoid-hMR complexes (Fig. 3 and Table 1). Dexamethasone bound to hGR with an affinity higher (K_d: 3.7 nM) than cortisol or aldosterone (K_d: 11–14 nM). There were also differences in the off-rates since aldosterone and cortisol dissociated much more rapidly from the hGR than did dexamethasone (Fig. 3 and Table 1). Thus, the order of potency of the steroids for stabilizing the steroid-hMR complexes (Aldo > F > Dex) is opposite to that observed for the hGR (Dex > 036:004 > F > Aldo).

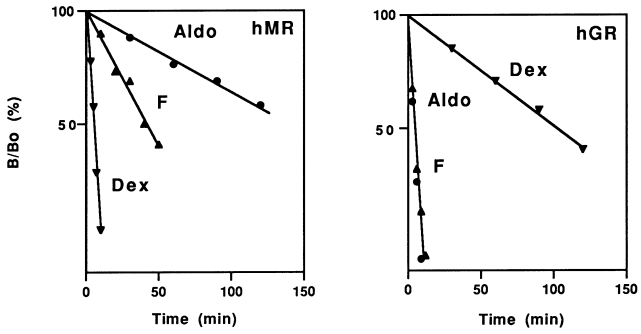

Fig. 3. Dissociation of corticosteroids from hMR and hGR. hMR and hGR were produced by translation in vitro. and incubated with 10 nM [^{3}H]steroid for 30 min at 20°C. The end of this incubation period was time zero for kinetic analysis. An aliquot was kept at 20°C to measure the stability of steroid-receptor complexes, and an other one was incubated with 10^{-6} M of the corresponding unlabeled steroid. Bound and free steroids were separated by dextran-charcoal. Non-specific binding was measured in parallel incubations for each incubation time. Results were corrected for receptor stability and are expressed as a percentage of the binding measured at zero time.

3.3. Specific corticosteroid hydroxyl groups determine hMR and hGR activities

Aldosterone, cortisol and DOC have two carboxyl groups in common at C3 and C20 and a hydroxyl group at C21. DOC is devoid of any other substituent, aldosterone has a 11–18 hemiketal bridge and cortisol has C11β and C17α hydroxyl groups (Fig. 1). The hMR and hGR transactivation functions in response to natural or synthetic steroids bearing a hydroxyl group at C11, C17 and/or C18 were measured. At 10^{-9} M DOC maximally stimulated hMR transactivation, but hMR was less sensitive to DOC (ED_{50}: 2×10^{-10} M) than to aldosterone (ED_{50}: 8×10^{-11} M) (Fig. 4). The presence of a 11β-hydroxyl group (corticosterone, B), or a 17α-hydroxyl group (cortexolone, S), or 11β- and 17α-hydroxyl groups (cortisol, F) led to a decrease of the steroid ability to stimulate hMR transactivation (ED_{50}: 5×10^{-9}–10^{-7} M). Dexamethasone with 11β-, 17α- and 21-hydroxyl groups, stimulated hMR transactivation with an ED_{50} of 2×10^{-8} M (Fig. 4). The effect of C18-hydroxylation on the mineralocorticoid activity was examined by testing the ability of 18-hydroxycortisol (18OHF) and 18-oxocortisol (18oxoF), two natural corticosteroids whose urinary excretion is increased during primary aldosteronism [24]. The hMR transactivation activity was very low in the presence of 18OHF, even when the steroid concentration was high. In contrast, 18oxoF was almost as potent as DOC in stimulating hMR transactivation (ED_{50}: 2×10^{-10} M).

Dexamethasone was the best stimulator of hGR transacti-

Table 1
Corticosteroid binding to hMR and hGR

	hMR		hGR	
	K_d (nM)	$t_{1/2}$ (min)	K_d (nM)	$t_{1/2}$ (min)
Aldosterone	0.52 ± 0.03	140	14.4 ± 2.1	5
Cortisol	0.49 ± 0.02	45	11.7 ± 0.8	5
Dexamethasone	0.73 ± 0.24	7	3.7 ± 0.6	100

hMR and hGR were synthesized in rabbit reticulocyte lysate. The lysate was diluted two-fold with TEGWD buffer and incubated with [^{3}H]steroid for 4 h at 4°C. Bound and free steroids were separated with dextran-charcoal and the dissociation constant at equilibrium (K_d) were determined. The half life ($t_{1/2}$) of the steroid–hMR and hGR complexes was determined from the experiments reported in Fig. 3.

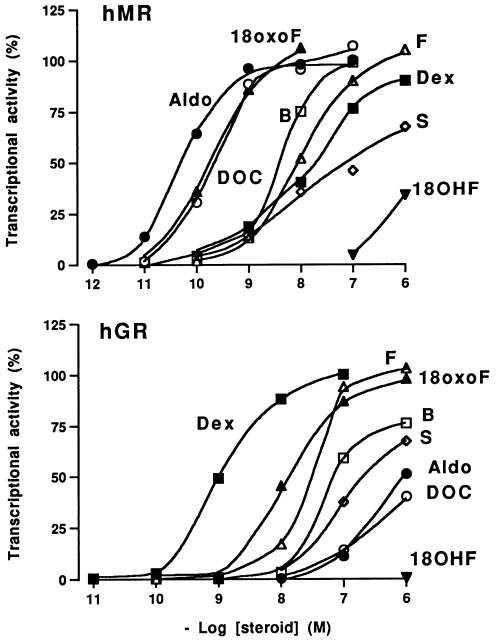

Fig. 4. Transactivation properties of hMR and hGR in response to various corticosteroids. COS-7 cells were transiently transfected with pchMR or pchGR, pFC31luc as reporter plasmid and a β-galactosidase internal reporter to correct for transfection efficiency. Cells were treated for 24 h with corticosteroids: aldosterone (Aldo), deoxycorticosterone (DOC), corticosterone (B), cortexolone (S), cortisol (F), 18-hydroxycortisol (18OHF), 18-oxocortisol (18oxoF), dexamethasone (Dex). hMR and hGR transactivation activities are determined by measuring luciferase activity, normalized to the internal β-galactosidase control and expressed as percent of the receptor activities in response to 10^{-9}M aldosterone (hMR) and 10^{-8}M dexamethasone (hGR). Each point is the mean of three separate experiments.

vation, whereas DOC, Aldo and 18OHF were very poor activators of the hGR function, even at 10^{-6} M. B and S were better than DOC, but 10^{-6} M did not produce the maximal dexamethasone-induced hGR response. 10^{-7}M F or 18oxoF fully stimulated hGR transactivation (Fig. 4).

Thus, DOC, which has the 'minimal' corticosteroid structure, has a high mineralocorticoid activity and a low glucocorticoid activity. The 11–18 hemiketal group slightly increased both mineralocorticoid and glucocorticoid activities, whereas hydroxyl groups at position 11, 17 and 18 dramatically decreased both activities. Hydroxylation at positions 11 and 17 decreased the mineralocorticoid activity, but enhanced the glucocorticoid activity.

4. Discussion

We have identified the corticosteroid hydroxyl groups responsible for selective hormonal recognition by each class of corticosteroid receptors.

The first step in the cascade of events that follows ligand binding to its receptor is a change in the receptor conformation. In agreement with previous reports [20–23], ligand binding to hMR or hGR produces a receptor compaction, with an increase in the resistance of the hMR 30 kDa fragment and the hGR 27 and 30 kDa fragment to proteolysis. This protection was depending upon the ligand concentration and ligand structure. Aldosterone that dissociates more slowly from hMR than glucocorticoids was the most efficient in protecting the receptor against proteolysis, a result in good agreement with the observation that the unliganded receptor is more sensitive to proteolysis than the liganded receptor. Similarly dexamethasone, characterized by a slow off-rate from the hGR, was the most efficient to protect this receptor. Thus, the steroid ability to protect the receptor against proteolysis is depending upon its dissociation rate from the receptor.

The receptor activity was also depending upon the ligand concentration and ligand structure. Aldosterone and dexamethasone, which dissociate very slowly from hMR and hGR, respectively, are the best stimulators of the hMR and hGR transactivation function. The order of potency of the steroids to stabilize the receptors is the same as that to activate the receptor. Nevertheless the steroid concentrations required to ensure 50% of the maximal receptor protection against proteolysis are higher than those necessary to induce 50% of the maximal receptor activities in transactivation assays, suggesting that ligand-receptor complexes might exist under distinct conformational states. Analysis of the three dimensional structure of the ligand-free and agonist-bound LBD of several NRs has revealed that ligand binding induces structural modifications namely the folding back of the last helix (H12) towards the LBD core [25]. It has been proposed that the acquisition of a transcriptionally active conformation is a multistep process in which an intermediate inactive complex is first generated by ligand binding and then converted to an active conformational state [22,26]. As mineralocorticoid and glucocorticoid ligands are both able to induce a hMR and hGR compaction, it is likely that an intermediate conformational state exists for both receptors.

The results reported here show that the stability of the active steroid-receptor complexes is ensured by steroid-receptor contacts. DOC, which has the same C21 hydroxyl group as aldosterone and cortisol, but no substituent at C11, C17 and C18, is almost as effective as aldosterone in stimulating hMR transactivation. The contacts between hMR Asn-770 and the C21 hydroxyl group of corticosteroids, which are of crucial importance for the hMR activation [8], are probably sufficient to stabilize the active hMR conformation. The presence of one hydroxyl group at the C11 position (B) or C17 (S) or two hydroxyl groups at the C11 and C17 positions (F) in addition to the C21 hydroxyl group modifies the positioning of the steroids within the hMR ligand binding cavity in such a way that the steroid dissociates more rapidly from the receptor, making the stabilization of the active conformation possible only with high steroid concentrations. These results are compatible with steroid-docking studies that reveal that cortisol underwent a $\sim 40°$ rotation of cortisol around its C3–C17 axis within the hMR ligand binding pocket compared to aldosterone [8]. The presence of three hydroxyl groups at C11, C17 and C18 (18OHF) in addition to the C21 hydroxyl group causes a drastic decrease in mineralocorticoid activity, that could be due to the formation of a 18–20 hemiketal bridge preventing the steroid contacting Cys-942, a residue involved in the interaction with the C20 carboxyl group of mineralocorticoids [27]. In contrast, the presence of a 11–18 hemiketal group alone (aldosterone) or together with a 17β-hydroxyl group (18oxoF) increases the mineralocorticoid activity. This result is worth noting since elevated urinary and plasma concentrations of 18oxoF are features of primary dexamethasone-sensitive hyperaldosteronism [24].

The anchoring sites of the glucocorticoid hormones within

the GR-LBD have not yet been identified. Nevertheless, the common fold of the NRs [25] and the sequence similarities suggest that the C3-ketone function of the glucocorticoid hormones are anchored within the hGR-LBD by residues Gln-570 and Arg-611 (corresponding Gln-776 and Arg-817 in the hMR) and the C21 hydroxyl group by Asn-564, which corresponds to residue Asn-770 in the hMR. We propose that the contact between hGR and the C21 hydroxyl group of DOC, presumably through Asn-564, does not stabilize the active hGR conformation, and that additional contacts through the C11 and/or C17 hydroxyl group (corticosterone, cortexolone, cortisol) are necessary to enhance the stability of the active hGR conformation.

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