

EXPERIMENTAL STUDY

Agonistic and antagonistic properties of progesterone metabolites at the human mineralocorticoid receptor

M Quinkler, B Meyer, C Bumke-Vogt, C Grossmann, U Gruber, W Oelkers, S Diederich and V Bähr

Department of Endocrinology, Klinikum Benjamin Franklin, Freie Universität Berlin, Hindenburgdamm 30, 12200 Berlin, Germany

(Correspondence should be addressed to M Quinkler; Email: LepeniesQuinkler@compuserve.com)

Abstract

Objective: Progesterone binds to the human mineralocorticoid receptor (hMR) with nearly the same affinity as do aldosterone and cortisol, but confers only low agonistic activity. It is still unclear how aldosterone can act as a mineralocorticoid in situations with high progesterone concentrations, e.g. pregnancy. One mechanism could be conversion of progesterone to inactive compounds in hMR target tissues.

Design: We analyzed the agonist and antagonist activities of 16 progesterone metabolites by their binding characteristics for hMR as well as functional studies assessing transactivation.

Methods: We studied binding affinity using hMR expressed in a T7-coupled rabbit reticulocyte lysate system. We used co-transfection of an hMR expression vector together with a luciferase reporter gene in CV-1 cells to investigate agonistic and antagonistic properties.

Results: Progesterone and 11 β -OH-progesterone (11 β -OH-P) showed a slightly higher binding affinity than cortisol, deoxycorticosterone and aldosterone. 20 α -dihydro(DH)-P, 5 α -DH-P and 17 α -OH-P had a 3- to 10-fold lower binding potency. All other progesterone metabolites showed a weak affinity for hMR. 20 α -DH-P exhibited the strongest agonistic potency among the metabolites tested, reaching 11.5% of aldosterone transactivation. The agonistic activity of 11 β -OH-P, 11 α -OH-P and 17 α -OH-P was 9, 5.1 and 4.1% respectively. At a concentration of 100 nmol/l, progesterone, 17 α -OH-P and 20 α -DH-P inhibit nearly 75, 40 and 35% of the transactivation by aldosterone respectively. All other progesterone metabolites tested demonstrate weaker affinity, and agonistic and antagonistic potency.

Conclusions: The binding affinity for hMR and the agonistic and antagonistic activity diminish with increasing reduction of the progesterone molecule at C20, C17 and at ring A. We assume that progesterone metabolism to these compounds is a possible protective mechanism for hMR. 17 α -OH-P is a strong hMR antagonist and could exacerbate mineralocorticoid deficiency in patients with congenital adrenal hyperplasia.

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Introduction

The human mineralocorticoid receptor (hMR) regulates electrolyte transport across epithelia (1–5). hMR exhibits almost the same affinity for the mineralocorticoid (MC) aldosterone, the glucocorticoid cortisol and the progestogen progesterone (6–9). *In vivo*, cortisol is prevented from binding to hMR by the enzyme 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD-2), which converts cortisol to its inactive metabolite cortisone in hMR target cells (10). This enzyme-mediated specificity of hMR was first described by Funder *et al.* (11) and Edwards *et al.* in 1988 (12).

Progesterone shows a high affinity for hMR, but confers only a weak transactivation activity and is, therefore, an MC antagonist (13–15). During the luteal phase of the menstrual cycle, progesterone plasma concentrations range between 30 and 110 nmol/l. During

pregnancy the concentrations rise steadily until they peak at the end of the third trimester in the range 320–700 nmol/l (16, 17). In contrast, plasma aldosterone increases only slightly during the luteal phase and late pregnancy (0.6 and 5.8 nmol/l respectively) (18). Given the high binding affinity of progesterone for hMR (6, 7), it is not clear how aldosterone can maintain its function as an effective MC agonist in the presence of high concentrations of progesterone. One protective mechanism *in vivo* is the strong binding of progesterone to plasma proteins: only 3% of progesterone is unbound, whereas 30% of plasma aldosterone is unbound. This produces still a 10-fold excess of free progesterone over free aldosterone. An additional second protective mechanism could be a potent and effective metabolism of progesterone in the human kidney (more than 40% conversion of 1 μ mol/l progesterone), which could be equivalent to

the inactivating metabolism of cortisol to cortisone by 11 β -HSD-2 (19, 20). The conversion of progesterone to 17-hydroxylated, 20 α -reduced and ring A-reduced metabolites in an hMR target tissue could result in an enzyme-mediated protection of hMR from progesterone.

There is growing evidence that metabolites of steroids, e.g. of progesterone, are not totally inactive but may have significant biological effects. 3 α ,5 α -Tetrahydro-progesterone (3 α ,5 α -TH-P) and 3 α ,5 β -TH-P are ligands for the gamma-aminobutyric acid_A receptor and have anesthetic, anxiolytic and anti-convulsant potency (21–24). 5 β -Dihydro(DH)-P reduces myometrial contractions (25) and shows pyrogenic effects (21). 5 α -DH-P stimulates cell growth in a mammary tumor cell line and reduces tumor cell adhesion, whereas 3 α -DH-P inhibits tumor cell growth (26). There is additional evidence that the effect of 3 α -DH-P may be mediated by a membrane-bound receptor (27).

To support our hypothesis that hMR is protected from progesterone by enzymatic inactivation of progesterone, we studied transactivation and inhibition of the hMR by progesterone metabolites. For the most part only reports on binding studies in animals have been published so far: 17 α -OH-P was tested on the renal MR of rats (28, 29) and sheep (30), and 17 α -OH,20 α -DH-P on the sheep renal MR (30, 31). The 17-hydroxylated metabolites of progesterone have been of special interest because they increase blood pressure in adrenalectomized sheep (30, 31). On the other hand, progesterone and its metabolites are suspected of exacerbating MC deficiency in patients with congenital adrenal hyperplasia (CAH) (28, 32) by antagonism at the hMR.

All of these metabolites have not been studied for their intrinsic activity at the hMR. Rupprecht *et al.* (6) studied only transactivation activity and binding properties of 3 α ,5 α -TH-P at the hMR.

We examined the agonistic and antagonistic activity of several progesterone metabolites by co-transfecting an hMR expression vector and a luciferase reporter gene in CV-1 cells. The binding characteristics of progesterone metabolites to the hMR were examined in ligand-binding assays using an hMR expressed in a reticulocyte lysate system.

Materials and methods

Chemicals

The following nonradioactive steroids were purchased from Sigma Chemical Co. (St Louis, MO, USA): cortisol, aldosterone, progesterone (4-pregnen-3,20-dione), deoxycorticosterone (DOC), 20 α -DH-P (4-pregnen-20 α -ol-3-one), 5 α -DH-P (5 α -pregnan-3,20-dione), 3 β ,5 α -TH-P (5 α -pregnan-3 β -ol-20-one), 20 α -DH,5 α -DH-P (5 α -pregnan-20 α -ol-3-one), 16 α -OH-P (4-pregnen-16 α -ol-3,20-dione), 5 β -DH-P (5 β -pregnan-3,20-dione),

20 α -DH,3 β ,5 α -TH-P (5 α -pregnan-3 β ,20 α -diol), 20 α -DH, 3 α ,5 α -TH-P (5 α -pregnan-3 α ,20 α -diol), 3 α ,5 α -TH-P (5 α -pregnan-3 α -ol-20-one), 11 α -OH-P (4-pregnen-11 α -ol-3,20-dione), 11 β -OH-P (4-pregnen-11 β -ol-3,20-dione), and 20 α -DH,3 α ,5 β -TH-P (5 β -pregnan-3 α ,20 α -diol). 17 α -OH-P (4-pregnen-17 α -ol-3-one) was purchased from Makor Chemicals Ltd (Jerusalem, Israel), 17 α -OH,20 α -DH-P (4-pregnen-17,20 α -diol-3-one) and 3 α ,5 β -TH-P (5 β -pregnan-3 α -ol-20-one) from Paesel & Lorei GmbH & Co. (Hanau, Germany) and 6 β -OH-P (4-pregnen-6 β -ol-3,20-diol) from Steroids Inc. (Wilton, NH, USA). [1,2,6,7-³H]aldosterone (50 Ci/mmol) was obtained from Amersham International (Amersham, Bucks, UK).

Plasmids

hMR expression vector pRShMR was kindly given to us by R M Evans (Salk Institute, San Diego, CA, USA). The reporter construct pMSG-Luc, which contains the MMTV promoter driving the firefly luciferase gene, was given to us by B Gellersen (Hamburg, Germany) and the pchMR vector with full-length hMR cDNA driven by the T7 promoter for expression in the rabbit reticulocyte lysate was provided by M-E Rafestin-Oblin (Paris, France).

Cell culture and transfection

CV-1 cells were purchased from The American Type Culture Collection (Manassas, VA, USA) and seeded at a density of 2.5×10^4 cells/well in 1 ml Dulbecco's minimal essential medium (DMEM) (Biochrom, Berlin, Germany), supplemented with 10% fetal calf serum (Biochrom), 100 U penicillin/ml (Biochrom), 100 μ g streptomycin/ml (Biochrom) and 2.5 μ g amphotericin B/ml (Squibb Pharma GmbH, Munich, Germany). They reached 80–90% confluence in a humidified atmosphere after 72 h with 5% CO₂ at 37 °C. Twenty-four hours before and after the transfection procedure, the cells (6×10^4 /well) were maintained in a medium with charcoal-stripped fetal calf serum. Cells were transfected using LipofectAMINE Plus Reagent (Life Technologies, Karlsruhe, Germany) as described by the manufacturers with 0.3 μ g hMR expression vector pRShMR, 0.15 μ g reporter gene vector pMSG-Luc and 0.02 μ g SV-40-driven renilla luciferase gene pRL-SV40 (Dual-Luciferase Reporter Assay System; Promega Corp., Madison, WI, USA). The 250 μ l assay was incubated for 3 h at 37 °C. Afterwards 250 μ l DMEM and 20% charcoal-stripped fetal calf serum were added.

Transactivation by steroids

After 21 h, the cells were rinsed twice with PBS and then incubated with various concentrations (10^{-11} – 10^{-6} mol/l; $n = 4$ for each concentration) of

the steroids to be tested, which were dissolved in DMEM containing 0.1% ethanol. The cells were harvested after 24 h of incubation, and the cell extracts were assayed for steroid-dependent firefly luciferase and renilla luciferase (Dual-Luciferase Reporter Assay System; Promega Corp.) with a luminometer from Berthold GmbH & Co. (Wildbad, Germany). Transactivation was calculated by the ratio of relative light units obtained by the steroid-dependent firefly luciferase and renilla luciferase. To examine hMR antagonistic properties of the steroids, the transfected cells were incubated with 10^{-9} mol/l aldosterone alone or with increasing concentrations of the steroids (10^{-11} – 10^{-6} mol/l) to be tested.

Coupled cell-free transcription and translation (TNT)

hMR was expressed *in vitro* using plasmids containing hMR cDNA and a T7 promoter (pchMR) in a rabbit reticulocyte lysate system for TNT (Promega Corp.). The system was incubated at 30 °C for 90 min according to the manufacturer's instructions.

Steroid-binding characteristics at equilibrium

In order to study the steroid-binding characteristics, the lysate was diluted after translation with 1 vol ice-cold buffer containing 20 mmol/l Tris-HCl pH 7.4, 1 mmol/l EDTA, 1 mmol/l dithiothreitol, 20 mmol/l sodium tungstate and 10% glycerol (33). Aliquots of 25 μ l per duplicate of the diluted translation medium were incubated with 5 nmol/l [3 H]aldosterone in the absence or presence of 5 μ mol/l unlabeled aldosterone on a rocker platform for 4 h at 4 °C in order to determine the amount of nonspecific binding. Further aliquots were incubated with 5 nmol/l [3 H]aldosterone and increasing concentrations (10^{-11} – 10^{-6} mol/l) of unlabeled steroids for competition experiments ($n = 2$).

Unbound steroids were separated from bound steroids by using dextran-coated charcoal (DCC): 1 ml DCC suspension (0.5%) was added to each aliquot (25 μ l), shaken for 7 min on ice and centrifuged at 14 000 *g* for 10 min at 4 °C.

Radioactivity was measured with a β -counter (Winspectral 1414; Perkin Elmer, Turku, Finland) by adding 10 ml Ultima Gold solution (Packard Bioscience, Groningen, The Netherlands) to the probe; d.p.m. values were calculated online using an external standard.

Statistical procedures

Scatchard analyses (34) were performed with eight concentrations of [3 H]aldosterone (0.1–12.8 nmol/l) in order to determine the affinity of aldosterone for hMR synthesized by the TNT method and to calculate the K_d value. K_i values of inhibitors were

calculated using the Cheng-Prusoff equation ($K_i = IC_{50}/(1 + ([^3\text{H-ald}]/K_d \text{ of } ^3\text{H-ald}))$) (35). Duncan's multiple range test was used for multiple comparisons of transactivation and inhibition experiments.

Results

Binding characteristics of progesterone metabolites to hMR

The binding affinities of progesterone and its metabolites for hMR were assessed by competition experiments using [3 H]aldosterone as specific ligand. The results are presented in Fig. 1A–D. Progesterone and 11β -OH-P showed a slightly higher binding affinity than cortisol, DOC and aldosterone. 20α -DH-P, 5α -DH-P and 17α -OH-P had a 3- to 10-fold lower binding potency. 5β -DH-P, $3\beta,5\alpha$ -TH-P, 11α -OH-P, 20α -DH, 5α -DH-P, 6β -OH-P and 17α -OH, 20α -DH-P displayed binding affinities (K_i) between 180 and 500 nmol/l. All other progesterone metabolites showed very low or no affinity for hMR in the range of concentrations tested (10^{-11} – 10^{-6} mol/l). It is noteworthy that 10^{-11} mol/l progesterone lowers the binding of 5 nmol/l [3 H]aldosterone by ~20%. Souque *et al.* (8) reported similar findings with a 15% reduction of [3 H]aldosterone binding at 5×10^{-10} mol/l progesterone. We tested lower progesterone concentrations (10^{-13} – 10^{-11} mol/l) for hMR binding in our system and measured a 9% reduction of [3 H]aldosterone binding at the lowest concentration increasing to 20% at 10^{-11} mol/l (data not shown). But Rupprecht *et al.* (6) found more than 80% reduction of [3 H]aldosterone binding at very low progesterone levels, suggesting that this massive reduction might represent an artifact (7). The order of potency and the K_i values reported in Table 1 indicate that reduction at positions C20 and C5 and hydroxylation at position C17 of progesterone reduce the affinity of hMR binding. Further reduction of the progesterone metabolites lowers the affinity even more. Surprisingly, 5α -DH-P showed a much higher affinity for hMR than did 5β -DH-P. A dissociation constant (K_d value) of 5.0 nmol/l for the binding of [3 H]aldosterone was determined by Scatchard analysis (data not shown). We did not test 20α -DH, $3\alpha,5\alpha$ -TH-P and 20α -DH, $3\alpha,5\beta$ -TH-P for their binding affinity due to the lack of binding affinity of $3\alpha,5\alpha$ -TH-P and $3\alpha,5\beta$ -TH-P and the observation that C20 reduction further lowers the binding affinity.

MR transactivation properties of progesterone metabolites

The agonistic properties of progesterone metabolites for hMR were investigated by testing the ability to induce gene expression. CV-1 cells were therefore co-transfected with hMR expression vector pRShMR, the

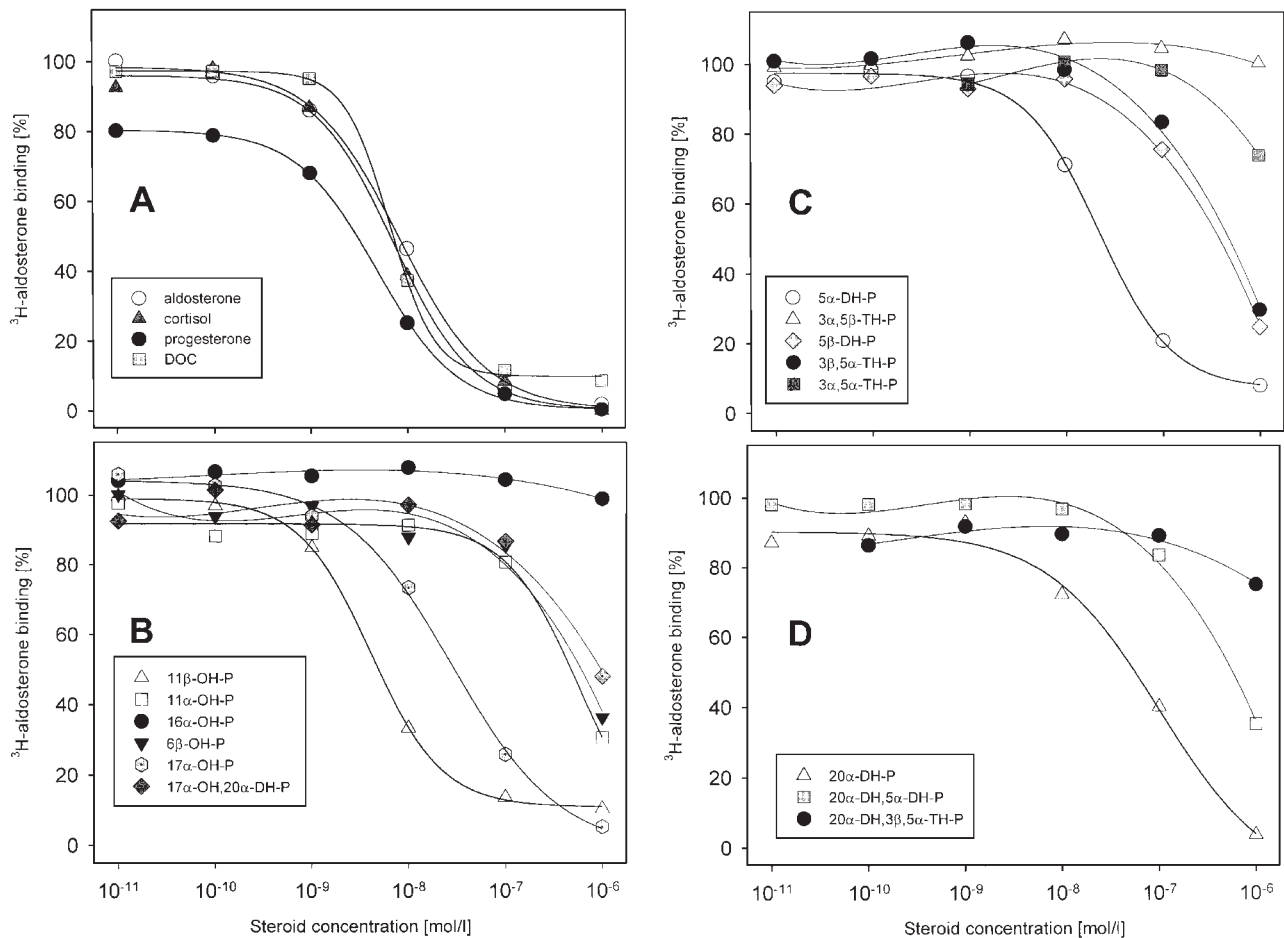


Figure 1 Competition of aldosterone, cortisol, progesterone and deoxycorticosterone (DOC) (A) and 14 progesterone metabolites (B–D) for [^3H]aldosterone binding to human mineralocorticoid receptor (hMR) in the TNT rabbit reticulocyte lysate. The lysate was incubated for 4 h at 4°C with 5 nmol/l [^3H]aldosterone and with increasing concentrations (10^{-11} – 10^{-6} mol/l) of unlabeled aldosterone, cortisol, DOC, progesterone or progesterone metabolites. Results are expressed as a percentage of the binding measured with [^3H]aldosterone alone. Data are means of $n = 2$ for each concentration. The K_i values calculated from these displacements are shown in Table 1.

reporter gene vector pMSG-Luc with MMTV promoter-driven firefly luciferase and the SV-40-driven renilla luciferase gene pRL-SV40. The transactivation was calculated by the ratio of light units produced by the steroid-dependent firefly luciferase and the steroid-independent renilla luciferase. The results are presented in Fig. 2A–D and Table 1. Aldosterone showed the strongest agonistic activity with 100% of the maximal transactivation at a concentration of 1 nmol/l. DOC with a low ED_{50} reached 70% of the transactivation observed with aldosterone and, in this system, reached a plateau agonist effect at 10^{-9} mol/l. Cortisol's agonistic activity was approximately ten times weaker than aldosterone's at comparable concentrations. Cortisol reached a plateau agonist effect at 10^{-8} mol/l. Progesterone showed relatively weak agonistic properties for hMR, reaching 27% of the maximal transactivation by aldosterone at a concentrations of 10^{-6} mol/l.

Among the progesterone metabolites, 20α -DH-P was the one with the strongest agonistic property, reaching 11.5% hMR transactivation. The 11-hydroxy metabolites of progesterone, 11β -OH-P and 11α -OH-P, reached 9 and 5.1% of aldosterone transactivation respectively. The data indicate that 11β -OH-P reaches a plateau level of transactivation at 10^{-9} mol/l (Fig. 2B). 17α -OH-P was an even weaker agonist with 4.2% transactivation. All other tested metabolites showed transactivation less than 3% of that of aldosterone (Figs. 2B–D and 4).

Inhibitory potency of progesterone metabolites for hMR

The antagonistic potency of progesterone metabolites for hMR was investigated in transfected CV-1 cells that were incubated with 10^{-9} mol/l aldosterone. The

Table 1 K_i values obtained from binding assays with various potential ligands for hMR (shown in Fig. 1A–D) and half-maximal response values (ED_{50}) derived from dose–response curves shown in Fig. 2A–D. The IC_{50} values were calculated from inhibition experiments presented in Fig. 3A–D. ‘> 1000’ = no binding affinity and no ED_{50} or IC_{50} values could be calculated in the tested range of concentrations (10^{-11} – 10^{-6} mol/l).

	K_i (nmol/l)	ED_{50} (nmol/l)	IC_{50} (nmol/l)
Progesterone	1.2	>1000	11.0
11 β -OH-P	2.4	>1000	>1000
Cortisol	3.0	0.40	30.7
DOC	3.4	0.05	>1000
Aldosterone	3.5	0.04	—
20 α -DH-P	10.8	>1000	282.5
5 α -DH-P	12.8	>1000	483.0
17 α -OH-P	16.5	>1000	135.2
5 β -DH-P	188.5	>1000	589.1
3 β , 5 α -TH-P	242.5	>1000	436.5
11 α -OH-P	256.5	>1000	776.2
20 α -DH, 5 α -DH-P	303.6	>1000	>1000
6 β -OH-P	305.5	>1000	555.9
17 α -OH, 20 α -DH-P	500.5	>1000	>1000
16 α -OH-P	>1000	>1000	>1000
3 α , 5 α -TH-P	>1000	>1000	>1000
3 α , 5 β -TH-P	>1000	>1000	>1000
20 α -DH, 3 β , 5 α -TH-P	>1000	>1000	>1000
20 α -DH, 3 α , 5 α -TH-P	Not tested	>1000	>1000
20 α -DH, 3 α , 5 β -TH-P	Not tested	>1000	>1000

results are shown in Figs. 3A–D and 4. The IC_{50} values are presented in Table 1. Progesterone demonstrated a high antagonistic potency. At a concentration of 100 nmol/l progesterone, nearly 75% of the trans-activation by aldosterone was inhibited. 17 α -OH-P and 20 α -DH-P were weaker antagonists, but 100 nmol/l still inhibited more than 40 and 35% respectively of the transactivation by aldosterone (Figs. 3B and D and 4). 20 α reduction of 17 α -OH-P diminishes the antagonistic effect (Fig. 3B). The metabolites 5 α -DH-P and 5 β -DH-P (Fig. 3C) showed less but still measurable antagonistic activity. Further reduction of these 5 α -DH- and 5 β -DH-metabolites at the 3 α -position diminishes the antagonistic potency completely. Surprisingly, 3 β -reduction of 5 α -DH-P showed no reduction in antagonistic potency (3 β , 5 α -TH-P in Fig. 3C). Besides 17 α -OH-P, the hydroxylated metabolites 6 β -OH-P and 11 α -OH-P display some antagonistic potency (Fig. 3B). It is noteworthy that 11 β -OH-P, binding with high affinity to the hMR, showed no antagonistic activity (Fig. 3B). All other progesterone metabolites tested demonstrated no antagonist influence at the hMR (Table 1; Figs. 3 and 4).

Discussion

During pregnancy, the plasma concentration of the hMR antagonist progesterone rises to very high levels that exceed those of aldosterone by a 100-fold.

This excess is diminished to 10-fold by the stronger plasma protein binding of progesterone. The anti-MC effect of progesterone is indicated *in vivo* by progressive activation of the renin–aldosterone system in normal pregnancy. Two other observations indicate the importance of the anti-MC progesterone: (i) our group observed several women with Addison’s disease during pregnancy, who had an increasing need for 9 α -fluoro-cortisol substitution as pregnancy advanced in order to keep blood pressure and serum potassium in the normal range (36); and (ii) in patients with primary hyperaldosteronism, serum potassium and blood pressure often normalized during pregnancy with recurrence of hypokalemia and hypertension after delivery (36, 37).

Since the *in vivo* anti-MC effect of progesterone seems to be moderate, we hypothesized that progesterone is metabolized by enzymes of hMR target tissues similar to the way cortisol is metabolized by 11 β -HSD-2 to protect the hMR (10, 19). We identified a potent and efficient enzyme system in male and in pre- and postmenopausal human kidneys (19, 20). Progesterone was converted efficiently even at high progesterone concentrations (10^{-6} mol/l) to 20 α -DH-P, 17 α -OH-P, 17 α -OH, 20 α -DH-P, 5 α -DH-P, 20 α -DH, 5 α -DH-P, 3 β , 5 α -TH-P and other ring A-reduced metabolites (19). In the present study we investigated binding affinity and antagonistic and agonistic potency of the progesterone metabolites at the hMR.

For both agonistic and antagonistic effects, binding to hMR is a prerequisite. Thus far hMR has not been crystallized. Concepts of the function of hMR are derived by analogy through molecular modeling with the crystal structure of the human progesterone receptor (38–42). In the ligand-binding domain (LBD) of hMR, site I (composed of helices H3 and H5) interacts with the steroid’s C3-ketone group common to agonists and antagonists. Site II (helices H3 and H11) anchors the D ring by several bonds, which specifies agonist–hMR binding. Due to agonist binding, the helices H12 and H3 bend back to the LBD, and through this conformational change they open binding sites for cofactors. The stability of helices H12 and H3 in this activated state is crucial to agonist activity (40, 41, 43). Mutations in the LBD can lead to a conformational change of hMR and to completely different receptor agonist and antagonist patterns, such as a constitutive activation of hMR and a strong activation by antagonists such as progesterone and spironolactone (44).

The reported K_d value of [3 H]aldosterone for hMR is ~ 0.8 – 3 nmol/l (1, 9, 45, 46). Many other studies have been done in either disrupted cells or whole cells. In our studies the K_d value of [3 H]aldosterone for hMR was calculated to be 5.0 nmol/l using a cell-free system. But this cell-free system (33) may not accurately reflect *in vivo* binding affinities, due to the fact that binding affinity is also defined by the interaction of the receptor

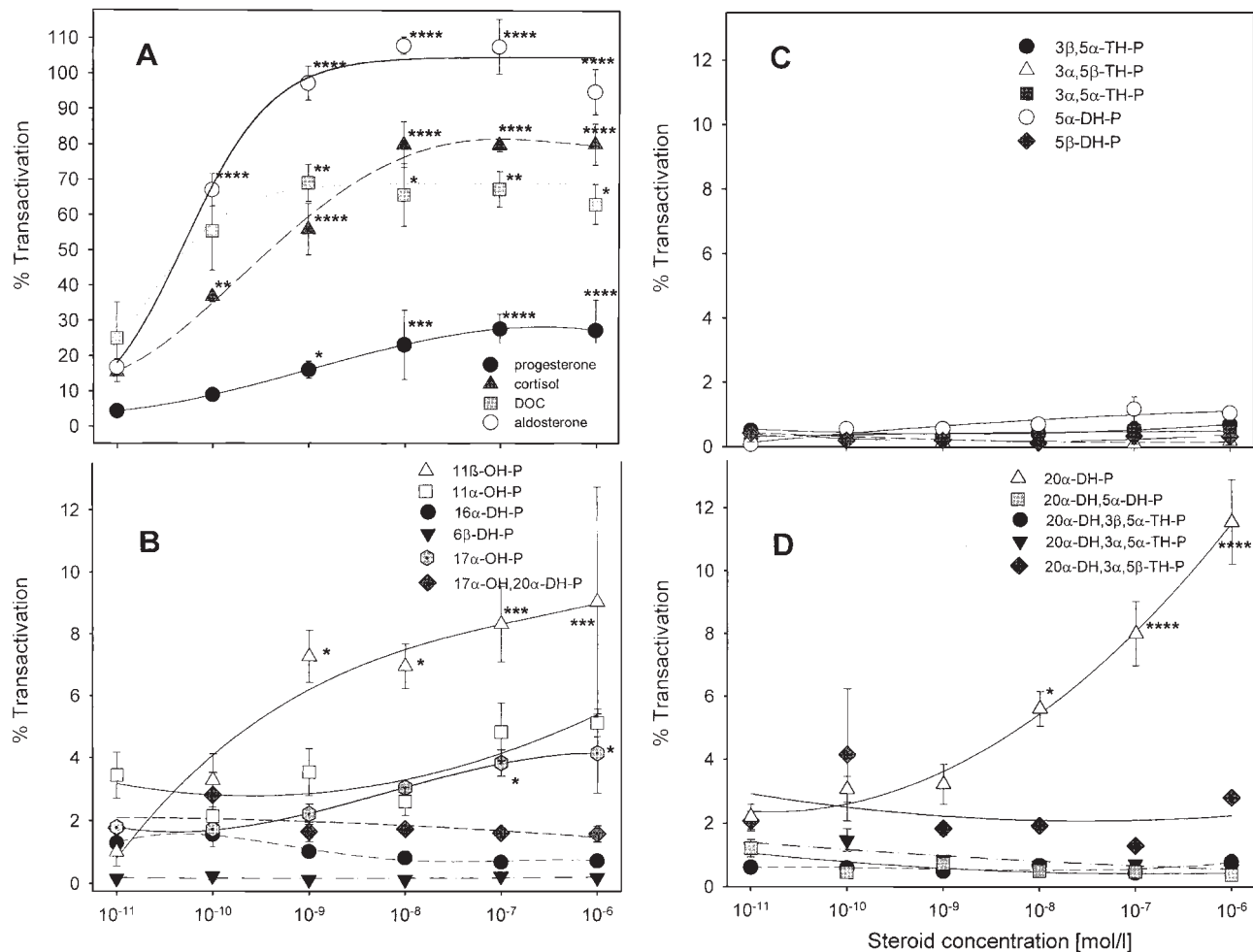


Figure 2 Transactivation properties of hMR in response to aldosterone, cortisol, progesterone, DOC (A) and 16 progesterone metabolites (B–D). CV-1 cells were transfected with pRShMR vector, pMSG-Luc and pRL-SV40. Transfected cells were treated for 22 h with aldosterone, progesterone, cortisol, DOC or progesterone metabolites in various concentrations (10^{-11} – 10^{-6} mol/l). The results are expressed as the ratio of firefly to renilla luciferase activity. The maximal transactivation activity of aldosterone was set as 100%. Data are means \pm S.E.M. ($n = 4$) for each concentration. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$ compared with 10^{-11} mol/l steroid concentration. Note the different scales of the y-axes of (A) and (B–D).

with the heat shock binding protein complex. Therefore, binding affinities must be interpreted carefully. There is no significant disassociation, though, for any of the compounds tested between binding affinity and either transactivation and/or inhibition of transactivation (Table 1). In addition the discrepancies in K_d and K_i values between laboratories may be due to different incubation times and the fact that lipophilic steroids, e.g. progesterone, are absorbed easily by plastic and glass material (3, 7, 8, 45).

Cortisol and hMR

Several groups found a similar affinity of aldosterone and cortisol for hMR (1, 6, 8, 9), and we confirmed these findings (Table 1; Fig. 1A). Some authors (40–42, 45) and ourselves reported a ~ 10 -fold weaker

transactivation activity of cortisol than aldosterone despite similar binding affinity (Table 1). But this different agonist affinity is at variance with other studies (6). The weaker transactivation of cortisol at the hMR (Fig. 2A) is probably due to distinct contacts involved in the interaction of hMR with aldosterone and cortisol. The 11–18 hemiketal group of aldosterone enhances the stabilization of the active hMR conformation, whereas the 11 β - or 17 α -hydroxyl groups of cortisol destabilize the active hMR conformation (38–41, 43). This destabilization of the ligand–receptor complex probably leads to a dissociation of cortisol from the hMR complex that is two to four times faster than that of aldosterone (1).

It is noteworthy that cortisol reached a plateau agonist effect at 10^{-8} mol/l (Fig. 2A), indicating also a possible partial antagonist effect (Fig. 3A). This plateau

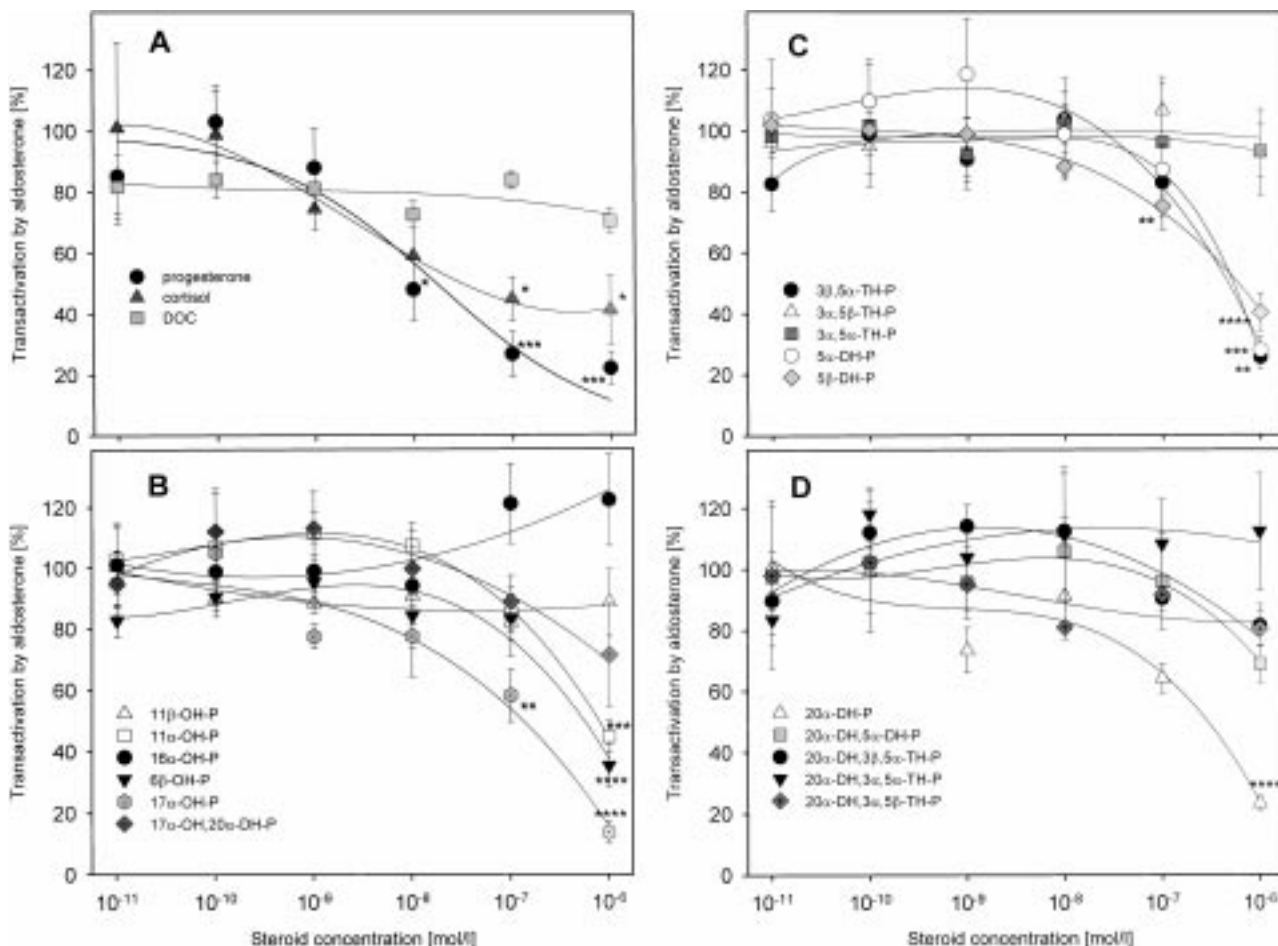


Figure 3 Inhibitory potency of cortisol, DOC, progesterone (A) and 16 progesterone metabolites (B–D) at the hMR. Transfected CV-1 cells were incubated with 10^{-9} mol/l aldosterone and increasing concentrations (10^{-11} – 10^{-6} mol/l) of cortisol, DOC, progesterone and progesterone metabolites. The results are expressed as the ratio of firefly and renilla luciferase activity. The maximal transactivation of aldosterone was set as 100%. Data are means \pm s.e.m. ($n = 4$) for each concentration. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.001$ compared with 10^{-11} mol/l steroid concentration.

agonist effect of cortisol is in agreement with the studies of Rogerson *et al.* (42). The significant antagonist properties at the hMR are surprising (Figs. 3A and 4) and could be due to the expression system used, due to a possible heterodimerization, e.g. interactions between cortisol and aldosterone liganded receptors, or due to multiple binding sites.

DOC and hMR

DOC, which has the same C21 group as aldosterone but lacks substituents at position C11, C17 and C18, shows a binding affinity similar to that of aldosterone (Table 1; Fig. 1A). DOC reached a plateau agonist effect at 10^{-9} mol/l, and showed a weaker transactivation activity ($\sim 70\%$) than aldosterone at higher concentrations (Table 1; Fig. 2A). A similar observation was made by Hellal-Levy *et al.* (47). This suggests, similar

to cortisol, a partial antagonist effect of DOC at the hMR. DOC showed $\sim 20\%$ of aldosterone inhibition of the range tested (Fig. 3A). DOC concentrations reach 0.2–0.3 nmol/l during the follicular and luteal phase, but they increase up to 1.8 nmol/l during pregnancy (16, 17), which could be a compensatory effect for the increasing concentrations of progesterone.

Progesterone and hMR

Several authors (6, 7) and ourselves found that progesterone binds with higher affinity to hMR than does aldosterone (Table 1; Fig. 1A). Arriza *et al.* (9) described a lower binding affinity for progesterone, but the incubation period was only 2.5 h, which is probably too short to achieve a steady state. Souque *et al.* (8) showed that progesterone has a low agonist MC activity with $\sim 24\%$ of the maximum aldosterone-induced

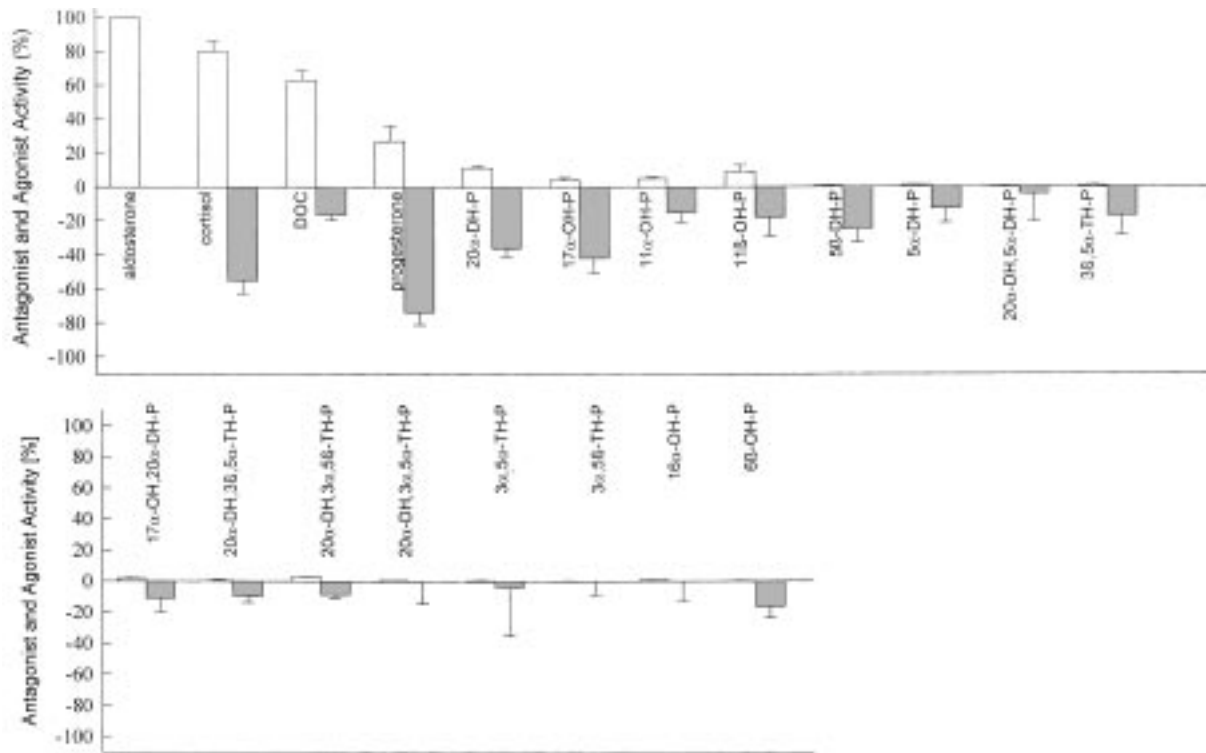


Figure 4 Effects of progesterone and its metabolites on transactivation of the hMR. The maximal agonist potential of the tested steroids at the hMR is shown as a percent (open bars); 100% = maximal hMR transactivation by aldosterone. The antagonist potential is shown as percent inhibition of aldosterone transactivation at a steroid concentration of 10^{-7} mol/l (gray bars). Data are means \pm S.E.M. ($n = 4$).

response at a progesterone concentration of 10^{-6} mol/l. We found a nearly identical agonist activity with 27% transactivation at 10^{-6} mol/l (Fig. 2A). In addition, the relatively weak *in vivo* anti-MC potency of progesterone compared with the strong hMR binding could be due to the instability of the progesterone–hMR complex. Progesterone is probably not able to form a strong bond to helix H12 and to induce the conformational change described for aldosterone hMR activation. This greater instability of the antagonist–hMR complex could lead to faster dissociation of progesterone from the receptor (8). The antagonistic potency of progesterone has been described with IC_{50} values ranging from 2 to 10 nmol/l (6, 33, 44). Our result, with a half-maximal inhibition of the aldosterone-induced activity of hMR by progesterone of 11 nmol/l (Table 1), is in agreement with these data.

17 α -OH-P, 17 α -OH, 20 α -DH-P and hMR

17-Hydroxylated metabolites of progesterone showed no or only a weak binding affinity for the rat and sheep MRs (28–31, 48). Recently, Geller *et al.* (44) found that at the hMR, 17 α -OH-P has a very low transactivation activity (less than 10% of the transactivation by aldosterone). In our experiments

17 α -OH-P showed a relatively high binding affinity for the hMR ($K_i = 16.5$ nmol/l), which was only a quarter of that of aldosterone (Table 1; Fig. 1B). The transactivation activity of 17 α -OH-P was very low, with 4.2% of the transactivation by aldosterone (Fig. 2B), whereas this steroid showed quite high antagonistic potency (Figs. 3B and 4; Table 1), with an IC_{50} of 135 nmol/l. Therefore, 17 α -OH-P is a potent hMR antagonist and not an agonist as suggested in former studies (30, 31). The serum concentration of 17 α -OH-P rises up to 29 nmol/l during the third trimester of pregnancy (49), and due to the presumably high binding affinity, 17 α -OH-P could play a role in hMR occupancy *in vivo*. The reduction at C20 to 17 α -OH, 20 α -DH-P greatly diminished the binding affinity (Fig. 1B) and the antagonist potency as well (Figs. 3B and 4; Table 1).

CAH is caused predominantly by 21-hydroxylase deficiency, which is an autosomal recessive disease. Mutations or deletions of the 21-hydroxylase gene cause glucocorticoid and often MC deficiency (32). In these patients, progesterone and 17 α -OH-P concentrations rise to very high levels. In patients with the simple virilizing, non-salt-losing form, 17 α -OH-P concentration reaches 70–240 nmol/l and in patients with the salt-losing form 400–1000 nmol/l (32, 50).

Regarding these high concentrations of 17 α -OH-P, the anti-MC potency of 17 α -OH-P, shown in this study, and the known antagonist effect of progesterone, give an explanation for the compensatory hyperaldosteronism in patients with the simple virilizing form (28, 51). The salt wasting is probably due to the complete lack of 21-hydroxylase activity and of aldosterone and cortisol production. Therefore, patients with this form can not compensate the anti-MCs 17 α -OH-P and progesterone by increasing aldosterone secretion (28). In addition the hMR antagonist 17 α -OH-P further exacerbates the MC deficiency in CAH.

5 α -DH-P, 5 β -DH-P and hMR

Differences in binding activity of 5 α - and 5 β -metabolites to the MR are explained on the basis of steroid planarity. A flat conformation of ring A and B of a steroid is essential for a strong binding to the MR. The flat 5 α -DH-P binds better to the rat MR than its bend isomer 5 β -DH-P (52), but they exhibit *in vitro* the same MC potency in the rat (53).

The properties of a flat steroid conformation is suggested also for other steroids: the flat metabolite 5 α -DH-aldosterone still possesses 1/30 and 5 α -DH-cortisol 1/500 of the aldosterone activity at the rat MR (54, 55). Interestingly, the flat steroid conformation seems to be more efficient for inhibitory potency for some enzymes, e.g. 5 α -reduced metabolites possess a higher inhibitory potency for 11 β -HSD-1 and -2 than do 5 β -reduced metabolites (56).

We showed that 5 α -DH-P had a ten times higher binding affinity for hMR than 5 β -DH-P (Fig. 1C). The influence of steroid planarity on the binding also plays a role at the hMR. The high binding affinity of 5 α -DH-P ($K_i = 12.8$ nmol/l) suggests that it could play a role *in vivo*, because concentrations of 5 α -DH-P rise to up to 29 nmol/l in the third trimester of pregnancy (57). 5 α -DH-P and 5 β -DH-P showed a similarly weak agonist and antagonist potency at the hMR. 5 β -DH-P probably plays no physiological role in hMR occupancy, because its serum concentrations reach only 2.3 nmol/l during pregnancy (57).

20 α -DH-P and hMR

20 α -DH-P is of special interest because it is one of the major progesterone metabolites in the human kidney (19, 20) and other organs (21). In addition, 20 α -DH-P reaches very high serum concentrations (up to 90 nmol/l) during the third trimester of pregnancy (49). 20 α -DH-P binds with higher affinity to hMR than does 17 α -OH-P and reaches 30% of the affinity of aldosterone (Fig. 1D; Table 1). 20 α -DH-P was the progesterone metabolite with the highest agonist activity and showed 11.5% of the transactivation by aldosterone (Fig. 2D). In contrast, the antagonist potency was similar to that of 17 α -OH-P (Fig. 3D;

Table 1). The serum concentrations of 20 α -DH-P indicate that this metabolite with weak agonist and considerable antagonist potency could play an important role in occupancy of the hMR *in vivo*, especially at the end of the third trimester of pregnancy.

11-, 6- and 16-hydroxylated progesterone metabolites and hMR

Until now, 11 β -OH-P had been tested only at the rat renal cytosolic MR and showed nearly 50% of the progesterone effect in displacing [³H]aldosterone from the receptor (29). In our study 11 α -OH-P and 11 β -OH-P displayed weak agonistic activity (5.1 and 9% respectively) (Fig. 2B) and showed an inhibitory potency at very high concentrations (Figs. 3B and 4). It is of considerable interest that 11 β -OH-P is capable of plateau levels of transactivation at 10⁻⁹ mol/l (Fig. 2B). This suggests a relatively good affinity for hMR, which is proven in our binding experiments (Fig. 1B). In contrast, 11 α -OH-P binds with lower affinity to hMR (Fig. 1B). There are no data available on concentrations of these metabolites during pregnancy, and, therefore, their physiological role remains unclear.

16 α -OH-P showed no binding affinity for hMR (Fig. 1B) and expressed no agonist or antagonist properties (Figs. 2B and 3B). 6 β -OH-P bound to hMR with an affinity similar to that of 11 α -OH-P and 17 α -OH,20 α -DH-P (Fig. 1B), but showed no agonist activity (Fig. 2B) and only a very weak antagonist activity at the hMR (Fig. 3B).

Ring A-reduced progesterone metabolites and hMR

Rupprecht *et al.* (6) showed that 3 α ,5 α -TH-P possesses a weak potency for displacing aldosterone from hMR. We confirmed this finding with our experiments (Fig. 1). We showed that the binding affinity for hMR was higher for 3 β ,5 α -TH-P than for 3 α ,5 α -TH-P and that 3 β ,5 α -TH-P had the strongest antagonistic activity among these ring A-reduced metabolites (Table 1). In general, ring A reduction and C20 reduction diminish the binding affinity of progesterone metabolites, and they display no agonistic and only very weak antagonistic activity. Serum concentrations during pregnancy (3 α ,5 α -TH-P reaches 13.6 nmol/l, 3 β ,5 α -TH-P 5 nmol/l and 3 β ,5 β -TH-P 2.2 nmol/l) (57) indicate that they probably have no *in vivo* effect on the hMR.

Effect of progesterone metabolites on other steroid metabolic pathways

The biological significance of progesterone metabolites *in vivo* is difficult to know with the techniques used. The final effect of these steroids would depend on many factors (e.g. plasma protein binding, transport

into the cell, intracellular binding, further metabolism) and not just on their antagonistic properties, but also on the effect they would have on other steroid metabolism.

For example 11 α - and 11 β -OH-P are very potent inhibitors of 11 β -HSD-2, comparable with glycyrrhetic acid and carbenoxolone (58–60). Therefore 11 α - and 11 β -OH-P can have significant hypertensiogenic properties as shown by Souness *et al.* (61). Their inhibitory potency on 11 β -HSD-1 is much weaker (56). 11 β -OH-P could also be converted by the action of 11 β -HSD-2.

Progesterone itself is a very potent inhibitor of 11 β -HSD-2 (19, 59). 5 α -DH-P, 20 α -DH-P, 3 β ,5 α -TH-P, 17 α -OH-P, 20 α -DH,5 α -DH-P and 17 α -OH,20 α -DH-P possess a much weaker inhibitory potency on 11 β -HSD-2 (19, 59). Some progesterone metabolites, e.g. 3 α ,5 β -TH-P and 17 α -OH,20 α -DH-P, show inhibitory potency on rat hepatic 5 β -reductase (60).

In addition it is noteworthy that progesterone has a binding affinity for the glucocorticoid receptor (6) and that progesterone metabolites might also exert glucocorticoid agonist and/or antagonist effects.

In conclusion, we have shown that the progesterone metabolites 20 α -DH-P and 17 α -OH-P, which are formed in the human kidney, exert antagonistic properties at the hMR due to their relatively strong binding affinity for hMR. They also have weak agonistic properties. The antagonistic potency of 17 α -OH-P could exacerbate MC deficiency in patients with CAH.

We showed that 11 β -OH-P binds with high affinity to the hMR. 5 α -DH-P binds better to hMR than does 5 β -DH-P, and both metabolites possess antagonistic activity as well. If progesterone metabolites are further reduced, the binding affinity and, consecutively, the agonistic and antagonistic activity is diminished. We suggest that progesterone metabolism to compounds with less agonistic and antagonistic potency at the hMR is a possible protective mechanism for the hMR.

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