3.2.1 Bindung und Transaktivierung von Progesteron und seinen Metaboliten am Mineralokortikoidrezeptor

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EXPERIMENTAL STUDY

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

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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 11β-OH-progesterone (11β-OH-P) showed a slightly higher binding affinity than cortisol, deoxycorticosterone and aldosterone. 20α-dihydro(DH)-P, 5α-DH-P and 17α-OH-P had a 3- to 10-fold lower binding potency. All other progesterone metabolites showed a weak affinity for hMR. 20α-DH-P exhibited the strongest agonistic potency among the metabolites tested, reaching 11.5% of aldosterone transactivation. The agonistic activity of 11β-OH-P, 11α-OH-P and 17α-OH-P was 9, 5.1 and 4.1% respectively. At a concentration of 100 nmol/l, progesterone, 17α-OH-P and 20α-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. 17α-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 11β-hydroxysteroid dehydrogenase type 2 (11β-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 μmol/l progesterone), which could be equivalent to

the inactivating metabolism of cortisol to cortisone by 11β-HSD-2 (19, 20). The conversion of progesterone to 17-hydroxylated, 20α-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. 3α,5α-Tetrahydro-progesterone (3α,5α-TH-P) and 3α,5β-TH-P are ligands for the gamma-aminobutyric $acid_A$ receptor and have anesthetic, anxiolytic and anticonvulsant potency (21–24). 5β-Dihydro(DH)-P reduces myometrial contractions (25) and shows pyrogenic effects (21). 5α-DH-P stimulates cell growth in a mammary tumor cell line and reduces tumor cell adhesion, whereas 3α-DH-P inhibits tumor cell growth (26). There is additional evidence that the effect of 3α-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: 17α-OH-P was tested on the renal MR of rats (28, 29) and sheep (30), and 17α-OH,20α-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 3α,5α-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), 20α-DH-P (4-pregnen-20α-ol-3-one), 5α-DH-P (5α-pregnan-3,20-dione), 3β,5α-TH-P (5α-pregnan-3β-ol-20-one), 20α-DH,5α-DH-P (5α-pregnan-20α-ol-3-one), 16α-OH-P (4-pregnen-16α-ol-3,20-dione), 5β-DH-P (5β-pregnan-3,20-dione), 20α-DH,3β,5α-TH-P (5α-pregnan-3β,20α-diol), 20α-DH, 3α,5α-TH-P (5α-pregnan-3α,20α-diol), 3α,5α-TH-P (5α-pregnan-3α-ol-20-one), 11α-OH-P (4-pregnen-11α-ol-3,20-dione), 11β-OH-P (4-pregnen-11β-ol-3,20-dione), and 20α-DH,3α,5β-TH-P (5β-pregnan-3α,20α-diol). 17α-OH-P (4-pregnen-17α-ol-3-one) was purchased from Makor Chemicals Ltd (Jerusalem, Israel), 17α-OH,20α-DH-P (4-pregnen-17,20α-diol-3-one) and 3α,5β-TH-P (5β-pregnan-3α-ol-20-one) from Paesel & Lorei GmbH & Co. (Hanau, Germany) and 6β-OH-P (4-pregnen-6β-ol-3,20-diol) from Steroloids Inc. (Wilton, NH, USA). [1,2,6,7-^{3}H]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 μg streptomycin/ml (Biochrom) and 2.5 μg amphotericin B/ml (Squibb Pharma GmbH, Munich, Germany). They reached 80–90% confluence in a humidified atmosphere after 72 h with 5% CO_2 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 μg hMR expression vector pRShMR, 0.15 μg reporter gene vector pMSG-Luc and 0.02 μg SV-40-driven renilla luciferase gene pRL-SV40 (Dual-Luciferase Reporter Assay System; Promega Corp., Madison, WI, USA). The 250 μl assay was incubated for 3 h at 37 °C. Afterwards 250 μl 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 μl per duplicate of the diluted translation medium were incubated with 5 nmol/l [^{3}H]aldosterone in the absence or presence of 5 μmol/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 μl), 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 ($K_i = IC_{50}/(1 + ([^3\text{H-aldo}]/K_d \text{ of } ^3\text{H-aldo}))$ (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, 3β,5α-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,3α,5α-TH-P and 20α-DH,3α,5β-TH-P for their binding affinity due to the lack of binding affinity of 3α,5α-TH-P and 3α,5β-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

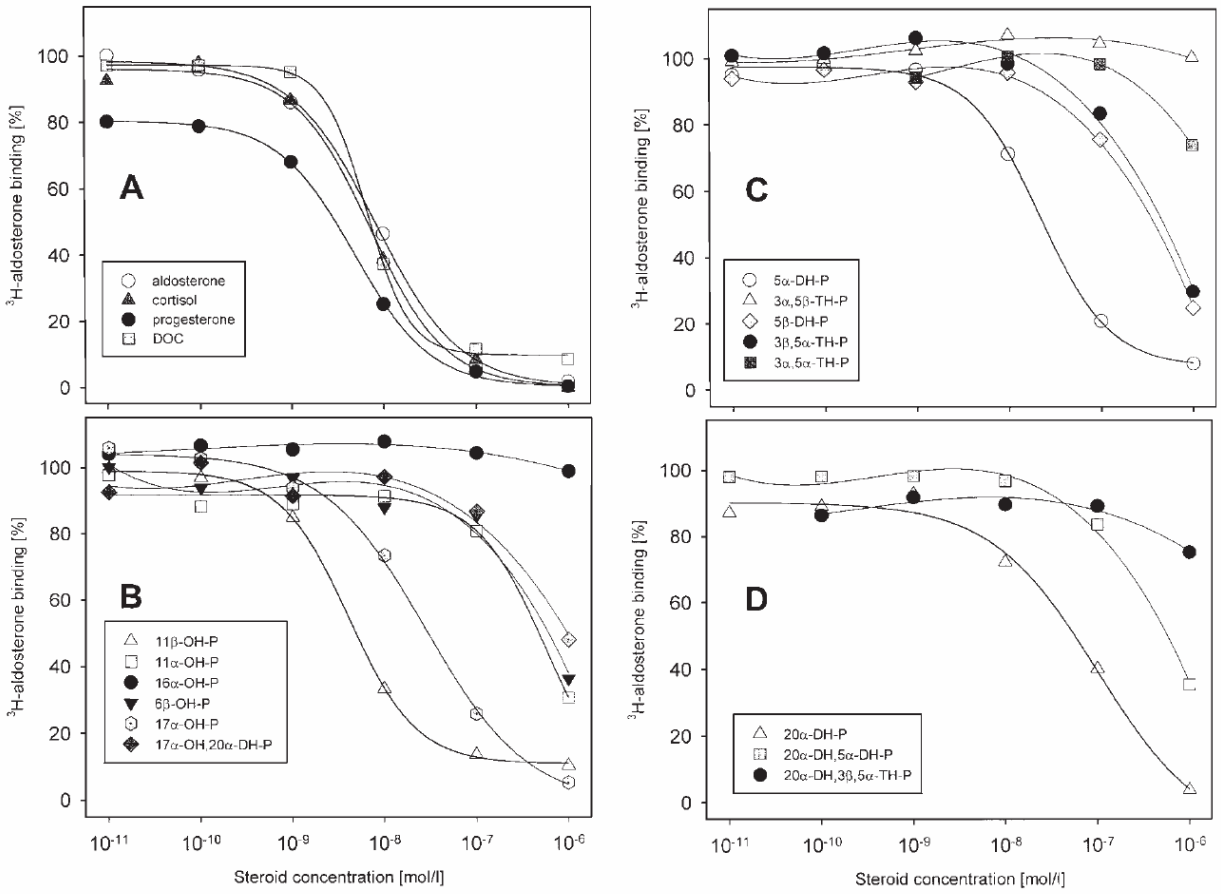

Figure 1 Competition of aldosterone, cortisol, progesterone and deoxycortisone (DOC) (A) and 14 progesterone metabolites (B–D) for [^{3}H]aldosterone binding to human mineraolcorticoid receptor (hMR) in the TNT rabbit reticulocyte lysate. The lysate was incubated for 4 h at 4 °C with 5 nmol/l [^{3}H]aldosterone 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 [^{3}H]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
11β-OH-P	2.4	>1000	>1000
Cortisol	3.0	0.40	30.7
DOC	3.4	0.05	>1000
Aldosterone	3.5	0.04	—
20α-DH-P	10.8	>1000	282.5
5α-DH-P	12.8	>1000	483.0
17α-OH-P	16.5	>1000	135.2
5β-DH-P	188.5	>1000	589.1
3β, 5α-TH-P	242.5	>1000	436.5
11α-OH-P	256.5	>1000	776.2
20α-DH, 5α-DH-P	303.6	>1000	>1000
6β-OH-P	305.5	>1000	555.9
17α-OH,20α-DH-P	500.5	>1000	>1000
16α-OH-P	>1000	>1000	>1000
3α,5α-TH-P	>1000	>1000	>1000
3α,5β-TH-P	>1000	>1000	>1000
20α-DH,3β,5α-TH-P	>1000	>1000	>1000
20α-DH,3α,5α-TH-P	Not tested	>1000	>1000
20α-DH,3α,5β-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 transactivation by aldosterone was inhibited. 17α-OH-P and 20α-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). 20α reduction of 17α-OH-P diminishes the antagonistic effect (Fig. 3B). The metabolites 5α-DH-P and 5β-DH-P (Fig. 3C) showed less but still measurable antagonistic activity. Further reduction of these 5α-DH- and 5β-DH-metabolites at the 3α-position diminishes the antagonistic potency completely. Surprisingly, 3β-reduction of 5α-DH-P showed no reduction in antagonistic potency (3β,5α-TH-P in Fig. 3C). Besides 17α-OH-P, the hydroxylated metabolites 6β-OH-P and 11α-OH-P display some antagonistic potency (Fig. 3B). It is noteworthy that 11β-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 9α-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 11β-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 20α-DH-P, 17α-OH-P, 17α-OH,20α-DH-P, 5α-DH-P, 20α-DH,5α-DH-P, 3β,5α-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

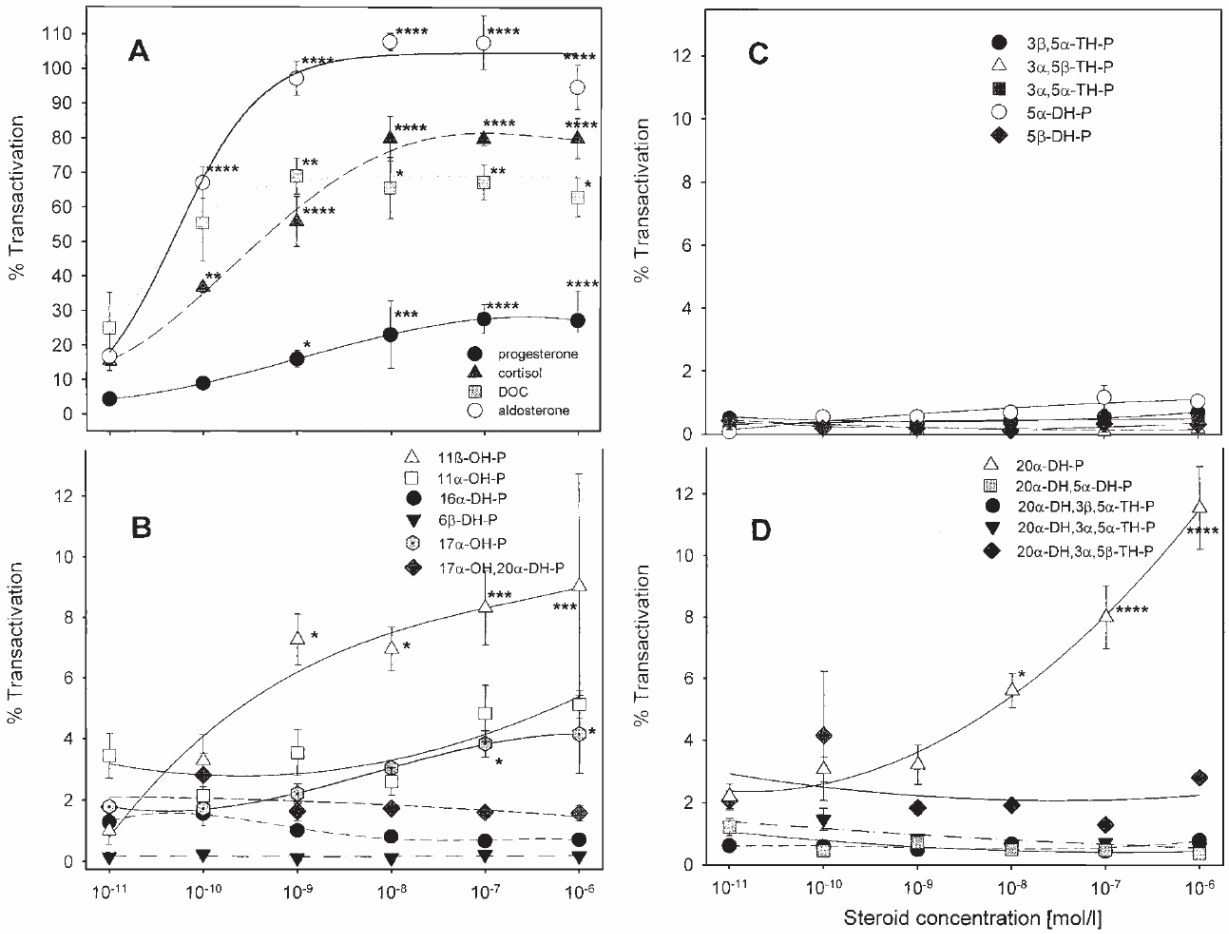

Figure 2 Transactivation properties of hMR in response to aldosterone, cortisol, progesterone, DOC (A) and 16 progesterone metabolites (B–D). CV-1 cells were transfected with pRShMR vector, pMSG-Luc and pRL-SV40. Transfected cells were treated for 22 h with aldosterone, progesterone, cortisol, DOC or 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±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 11β- or 17α-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

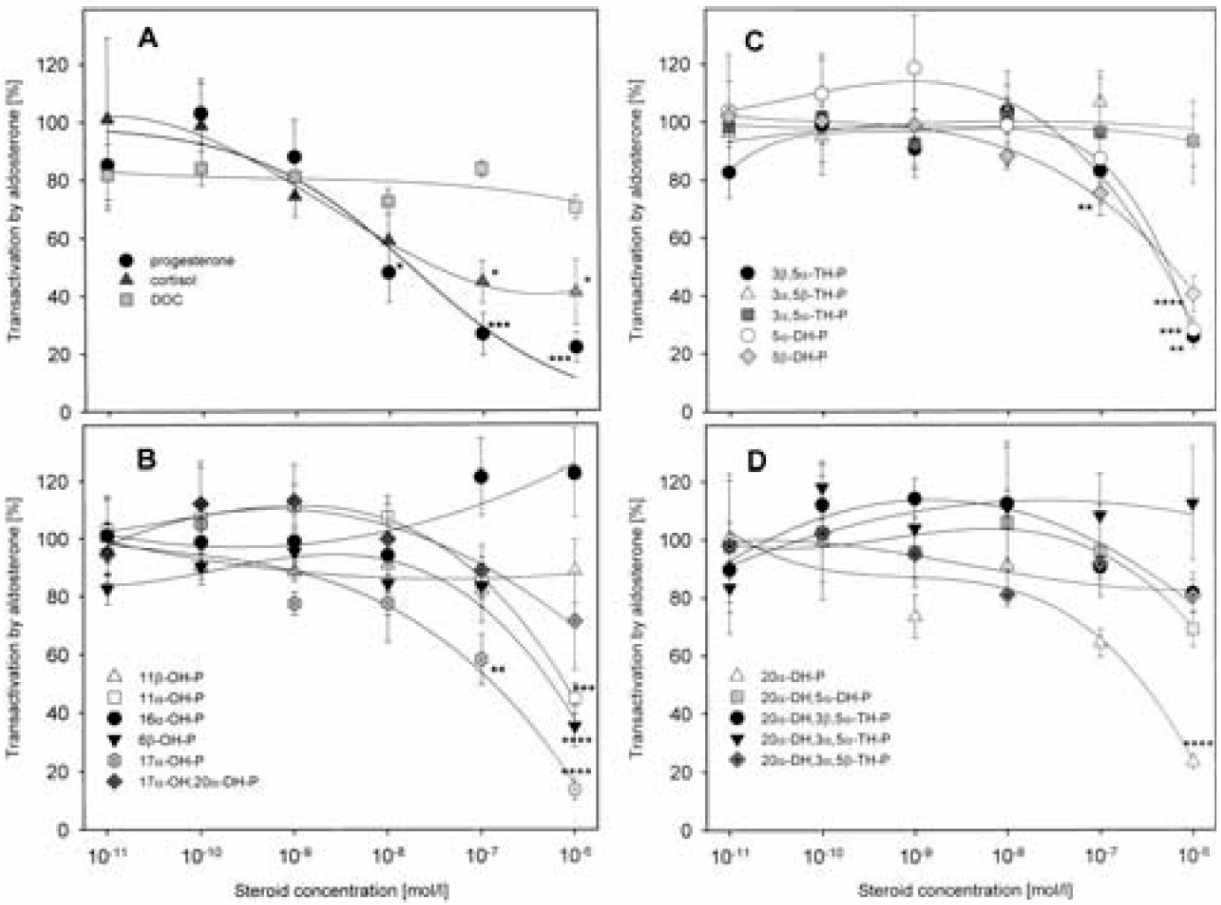

Figure 3 Inhibitory potency of cortisol, DOC, progesterone (A) and 16 progesterone metabolites (B–D) at the hMR. Transfected CV-1 cells were incubated with 10^{-9} mol/l aldosterone and increasing concentrations (10^{-11}–10^{-6} mol/l) of cortisol, DOC, progesterone and progesterone metabolites. The results are expressed as the ratio of firefly and renilla luciferase activity. The maximal transactivation of aldosterone was set as 100%. Data are means±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 (~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 ~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 ~24% of the maximum aldosterone-induced

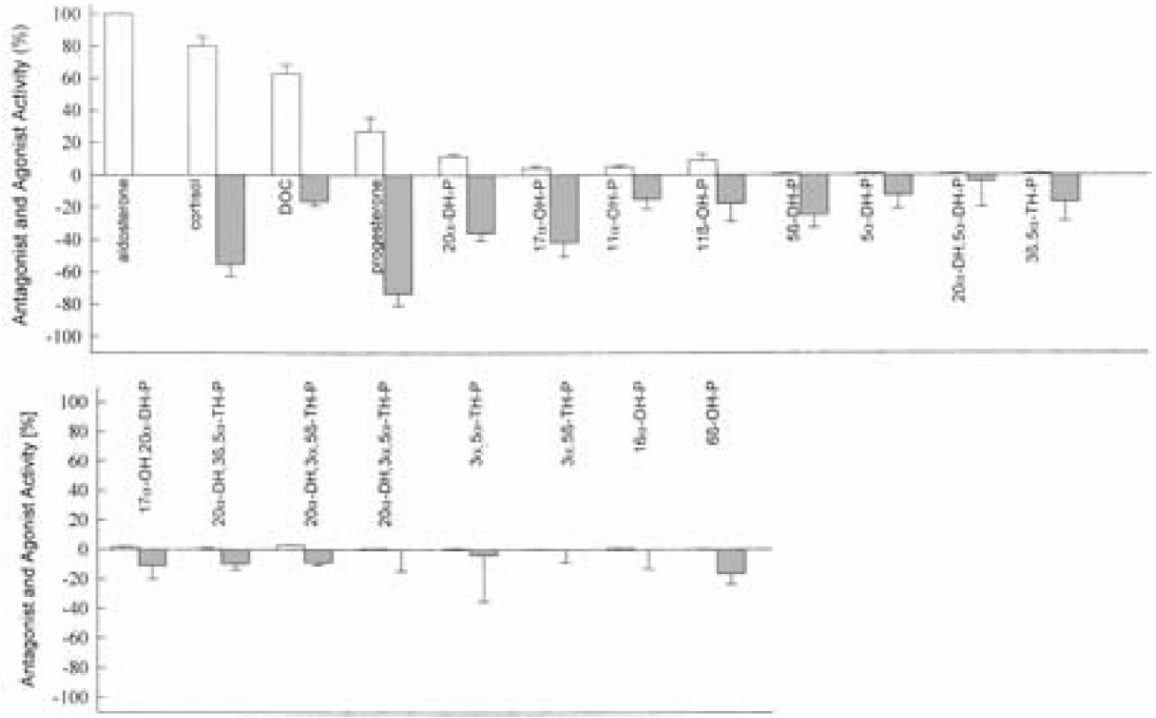

Figure 4 Effects of progesterone and its metabolites on transactivation of the hMR. The maximal agonist potential of the tested steroids at the hMR is shown as a percent (open bars); 100% = maximal hMR transactivation by aldosterone. The antagonist potential is shown as percent inhibition of aldosterone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

17α-OH-P, 17α-OH,20α-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, 17α-OH-P has a very low transactivation activity (less than 10% of the transactivation by aldosterone). In our experiments 17α-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 17α-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, 17α-OH-P is a potent hMR antagonist and not an agonist as suggested in former studies (30, 31). The serum concentration of 17α-OH-P rises up to 29 nmol/l during the third trimester of pregnancy (49), and due to the presumably high binding affinity, 17α-OH-P could play a role in hMR occupancy *in vivo*. The reduction at C20 to 17α-OH,20α-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 17α-OH-P concentrations rise to very high levels. In patients with the simple virilizing, non-salt-loosing form, 17α-OH-P concentration reaches 70–240 nmol/l and in patients with the salt-loosing form 400–1000 nmol/l (32, 50).

Regarding these high concentrations of 17α-OH-P, the anti-MC potency of 17α-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 17α-OH-P and progesterone by increasing aldosterone secretion (28). In addition the hMR antagonist 17α-OH-P further exacerbates the MC deficiency in CAH.

5α-DH-P, 5β-DH-P and hMR

Differences in binding activity of 5α- and 5β-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 5α-DH-P binds better to the rat MR than its bend isomer 5β-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 5α-DH-aldosterone still possesses 1/30 and 5α-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. 5α-reduced metabolites possess a higher inhibitory potency for 11β-HSD-1 and -2 than do 5β-reduced metabolites (56).

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

20α-DH-P and hMR

20α-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, 20α-DH-P reaches very high serum concentrations (up to 90 nmol/l) during the third trimester of pregnancy (49). 20α-DH-P binds with higher affinity to hMR than does 17α-OH-P and reaches 30% of the affinity of aldosterone (Fig. 1D; Table 1). 20α-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 17α-OH-P (Fig. 3D; Table 1). The serum concentrations of 20α-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, 11β-OH-P had been tested only at the rat renal cytosolic MR and showed nearly 50% of the progesterone effect in displacing [^{3}H]aldosterone from the receptor (29). In our study 11α-OH-P and 11β-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 11β-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, 11α-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.

16α-OH-P showed no binding affinity for hMR (Fig. 1B) and expressed no agonist or antagonist properties (Figs. 2B and 3B). 6β-OH-P bound to hMR with an affinity similar to that of 11α-OH-P and 17α-OH,20α-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 3α,5α-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 3β,5α-TH-P than for 3α,5α-TH-P and that 3β,5α-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 (3α,5α-TH-P reaches 13.6 nmol/l, 3β,5α-TH-P 5 nmol/l and 3β,5β-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 11α- and 11β-OH-P are very potent inhibitors of 11β-HSD-2, comparable with glycyrrhetinic acid and carbenoxolone (58–60). Therefore 11α- and 11β-OH-P can have significant hypertensiogenic properties as shown by Souness *et al.* (61). Their inhibitory potency on 11β-HSD-1 is much weaker (56). 11β-OH-P could also be converted by the action of 11β-HSD-2.

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

We showed that 11β-OH-P binds with high affinity to the hMR. 5α-DH-P binds better to hMR than does 5β-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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3.2.2 Wirkung von Progesteron *in vivo*

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Renal Inactivation, Mineralocorticoid Generation, and 11β-Hydroxysteroid Dehydrogenase Inhibition Ameliorate the Antimineralocorticoid Effect of Progesterone *in Vivo*

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Progesterone (P) is a strong mineralocorticoid receptor (MR) antagonist *in vitro*. The high P concentrations seen in normal pregnancy only moderately increase renin and aldosterone concentrations. In previous *in vitro* studies we hypothesized that this may be explained by intrarenal conversion of P to less potent metabolites. To investigate the *in vivo* anti-MR potency of P, we performed an infusion study in patients with adrenal insufficiency (n = 8). They omitted 9α-fluorocortisol for 4 d and hydrocortisone for 0.5 d before a continuous iv infusion of aldosterone for 8.5 h, with an additional iv P infusion commenced at 4 h. During aldosterone infusions the initially elevated urinary sodium to potassium ratio decreased significantly. Despite the 1000-fold excess of P over aldosterone, the urinary sodium to potassium ratio and urinary sodium excretion increased only slightly after 3 h of P infusion. We detected inhibition of renal 11β-hydroxysteroid dehydrogenase type 2 by P, thus giving cortisol/prednisolone access to the MR. Urinary and plasma concentrations of 17α-hydroxyprogesterone, a major metabolite of renal P metabolism, and those of serum androstenedione and deoxycorticosterone, a mineralocorticoid itself, increased significantly during P infusion. This supports the hypothesis of an effective protection of the MR from P by efficient extraadrenal downstream conversion of P. (*J Clin Endocrinol Metab* 88: 3767–3772, 2003)

PROGESTERONE (P) BINDS *in vitro* with higher affinity to the human mineralocorticoid receptor (MR) than aldosterone, but confers only low agonistic MR activity. Therefore, P is a MR antagonist *in vitro* (1–3). During pregnancy, P concentrations reach high levels [400–700 nmol/liter (125–220 ng/ml)] and by far exceed those of aldosterone [1–6 nmol/liter (0.36–2.16 ng/ml)]. The reason why aldosterone can still act as a potent mineralocorticoid in these situations is not fully understood. The 10-fold higher plasma protein binding of P compared with aldosterone (4) and the higher stability of the aldosterone-MR complex (5) may be contributing factors. However, we have previously shown that renal P metabolism could be an effective protective mechanism for the MR, similar to the protection by 11β-HSD type 2 (6, 7). This protective mechanism implies that during high P concentrations little anti-MR effect should be seen. This is in accordance with the progressive plasma volume expansion in normal pregnant women that is required for optimal pregnancy outcome. The anti-MR effect of P *in vivo* is demonstrated by activation of the renin-aldosterone system in normal pregnancy. Two additional observations indicate the anti-MR potency of progesterone. Firstly, pregnant women with Addison's disease have an increasing requirement for 9α-fluorocortisol substitution as pregnancy advances to maintain blood pressure and serum potassium in the normal range (8). Secondly, in patients with primary hyperaldosteronism, serum potassium and blood pressure often normalize during pregnancy, with recurrence of hypokalemia and hypertension after delivery (8, 9).

Until now the exact *in vivo* anti-MR potency of P was not known. Therefore, we sought to investigate its anti-MR potency *in vivo*, studying patients without functioning adrenal glands and thus devoid of endogenous aldosterone production. By choosing only males and postmenopausal females we ruled out the ovaries as a major source of endogenous P production. We challenged the patients with a continuous aldosterone infusion and then sought to antagonize the MR effect by P infusion.

Subjects and Methods

Subjects

Eight patients, aged 38–62 yr, without functioning adrenals (three postmenopausal women and five men) were recruited for the study (Table 1). Their renal function parameters and serum albumin levels were normal. They received detailed information on all aspects of the protocol and gave written informed consent before inclusion. The study protocol was reviewed and approved by the ethics committee of the Universitätsklinikum Benjamin Franklin (Berlin, Germany).

Study protocol

The patients stopped taking 9α-fluorocortisol 4 d before the study and hydrocortisone 0.5 d before the study. To prevent hypocortisolemic crisis the patients received 1 mg prednisolone at 0800 h on the study day, followed by 0.5 mg prednisolone every 2 h as requested by the ethics committee. We chose prednisolone as the glucocorticoid substitute because it possesses a weaker mineralocorticoid activity than cortisol. Dehydroepiandrosterone (DHEA) and estrogen/progestin replacement was stopped at least 1 wk before the study, whereas all other medication was continued. During the study patients were encouraged to drink 250 ml water/h to produce sufficient amounts of urine. The patients received

Abbreviations: DHEA, Dehydroepiandrosterone; 4-dione, androstenedione; DOC, deoxycorticosterone; MR, mineralocorticoid receptor; 17α-hydroxy-P, 17α-hydroxyprogesterone; P, progesterone.

TABLE 1. Characterization of eight patients without functioning adrenal glands

Patient	Etiology of adrenal insufficiency	Duration of disease (yr)	Sex	Age (yr)	BMI
I.B.	Bilateral adrenalectomy due to bilateral hyperplasia and hypercortisolism	10	F	53	20.0
K.S.	Autoimmune adrenalitis	11	F	57	23.6
U.W.	Autoimmune adrenalitis	4	M	37	23.9
M.K.	Autoimmune adrenalitis	30	F	62	20.8
K.W.K.	Bilateral adrenalectomy due to Cushing's syndrome	39	M	45	30.9
P.W.	Tuberculosis	31	M	57	28.4
N.W.	Bilateral adrenalectomy due to Cushing's syndrome	24	M	39	28.7
B.B.	Autoimmune adrenalitis	4	M	38	21.6

BMI, Body mass index; f, female; m, male.

an indwelling cannula into the antecubital vein of each arm. One cannula was used for aldosterone and P infusions, the other for blood sampling. The patients received a continuous infusion (Infusomat, Braun Ag, Melsungen, Germany) with aldosterone iv over 8.5 h (12.5 μg/h) starting at 0800 h. After 4 h a continuous P infusion was started (0.15 mg/kg·h for 90 min, followed by 0.65 mg/kg·h for 180 min). Blood sampling and assessment of urinary volume were performed every 30 min for 8.5 h. Heart rate and blood pressure were recorded every 2 h.

Preparation of infusion solutions

For aldosterone infusions, 150 μg lyophilized aldosterone (Clinalfa AG, Läufelfingen, Switzerland) were diluted in 500 ml 5% glucose Ringer's solution (containing 147.2 mmol/liter sodium, 4.02 mmol/liter potassium, 2.24 mmol/liter calcium, and 155.7 mmol/liter chloride). The P (Merck KgaA, Darmstadt, Germany) infusions were prepared as described previously (10, 11) with minor modifications. P (4.4 g) was dissolved in 220 ml ethanol solution (90%) and filtered under sterile conditions. Five milliliters of the P ethanol solution were injected slowly under constant shacking into the infusion solution containing 107.5 ml 5% glucose solution (Glucosteril Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany), 107.5 ml physiological electrolyte solution (Sterofundin 1/1E, Braun Melsungen AG, Melsungen, Germany), and 35 ml human 20% albumin solution (DRK Blutspendedienst Niedersachsen GmbH, Springe, Germany).

Analytical measurements

Serum hormone measurements were performed by RIA using commercially available assays: aldosterone (Diagnostic Products, Los Angeles, CA), P, 17α-hydroxyprogesterone (17α-hydroxy-P), estrone, testosterone, DHEA, and androstenedione (4-dione; all from DSL, Sinsheim, Germany). Cross-reactivity was less than 6% for all relevant steroids. Urinary excretion of 17α-hydroxy-P in urine was also measured by RIA after extraction as described previously (12). Plasma renin concentrations were measured using a Renin-IRMA kit (Nichols Institute Diagnostics, San Juan Capistrano, CA) (13). Plasma deoxycorticosterone (DOC) concentrations were determined at the Pharmacological Institute (Ruprechts Karl Universität, Heidelberg, Germany) using an extraction method with slit column chromatography, followed by RIA. Urinary measurements of prednisolone and prednisone were performed by HPLC analysis at Krankenhaus Spandau (Berlin, Germany). Serum measurements of sodium, potassium, albumin, creatinine, and urinary creatinine were measured using a Hitachi 917 analyzing machine (Hitachi Medical Systems GmbH, Wiesbaden, Germany) in the central laboratory of University Hospital UKBF. Urinary measurements of sodium and potassium were performed with an IL 943 analyzing machine (Instrumentation Laboratory GmbH, Kirchheim bei München, Germany). Urinary chloride was measured with a dilution kit using a CRT-10 analyzing machine (Nova Biomedical Corp., Waltham, MA). All samples from an individual were analyzed in a single assay.

Statistics

Results are expressed as the mean ± SEM. Statistical significance was taken as $P < 0.05$. Statistical analysis was performed with the Wilcoxon test. Due to the small number of patients (n = 8), the highest level of statistical significance that could be reached with this test was $P = 0.012$.

Results

We investigated the effect of aldosterone and P infusion on urinary electrolyte excretion as an expression of MR activation or inhibition. At the beginning of the study the patients presented with plasma aldosterone concentrations below 0.06 nmol/liter (0.0216 ng/ml; Fig. 1A) and were in a slightly hypomineralocorticoid status, indicated by the increased urinary sodium to potassium ratio (Fig. 2A). Under continuous aldosterone infusion the patients obtained normal, nonpregnant, plasma aldosterone concentrations [0.2–1 nmol/liter (0.072–0.360 ng/ml)] over the entire period of the study (Fig. 1A). Due to the aldosterone infusions, the urinary sodium to potassium ratio decreased significantly ($P < 0.05$; Fig. 2A), mainly because of significantly ($P < 0.05$) decreased sodium excretion (data not shown). Urinary chloride excretion (Fig. 2B) and plasma renin concentration did not change significantly during this period. Urinary volume increased significantly ($P < 0.05$) to approximately 140 ml/30 min during this period (Fig. 2C).

After 4 h the P infusion was started. Thirty to 60 min after starting the P infusion, all patients reported mild sleepiness. Plasma P (Fig. 1B) and 17α-hydroxy-P (Fig. 1C) concentrations increased slightly, but significantly ($P < 0.05$), during the first phase of P infusion. During the second concentration step plasma P and plasma 17α-hydroxy-P showed a more pronounced increase, thus reaching plasma P levels similar to those during the third trimester (Fig. 1, B and C). Considering the high plasma protein binding of P, only 25–40 nmol/liter (7.9–12.6 ng/ml; 3–5%) will be unbound in the circulation and accessible for enzymatic conversion. It appears that 35–60% of this unbound P is converted to 17α-hydroxy-P, resulting in an increased 17α-hydroxy-P plasma concentration [16 ± 2.6 nmol/liter (5.3 ± 0.9 ng/ml)]. In addition, the highly increased urinary 17α-hydroxy-P excretion during P infusion (3.5 nmol/min) may be based on glomerular filtration (~1.6 nmol/min) as well as on renal production.

The urinary sodium to potassium ratio (Fig. 2A) and sodium excretion increased significantly ($P < 0.05$) during P infusion, but the urinary sodium to potassium ratio did not reach similar hypomineralocorticoid levels as at the beginning of the study. Urinary chloride excretion (Fig. 2B), uri-

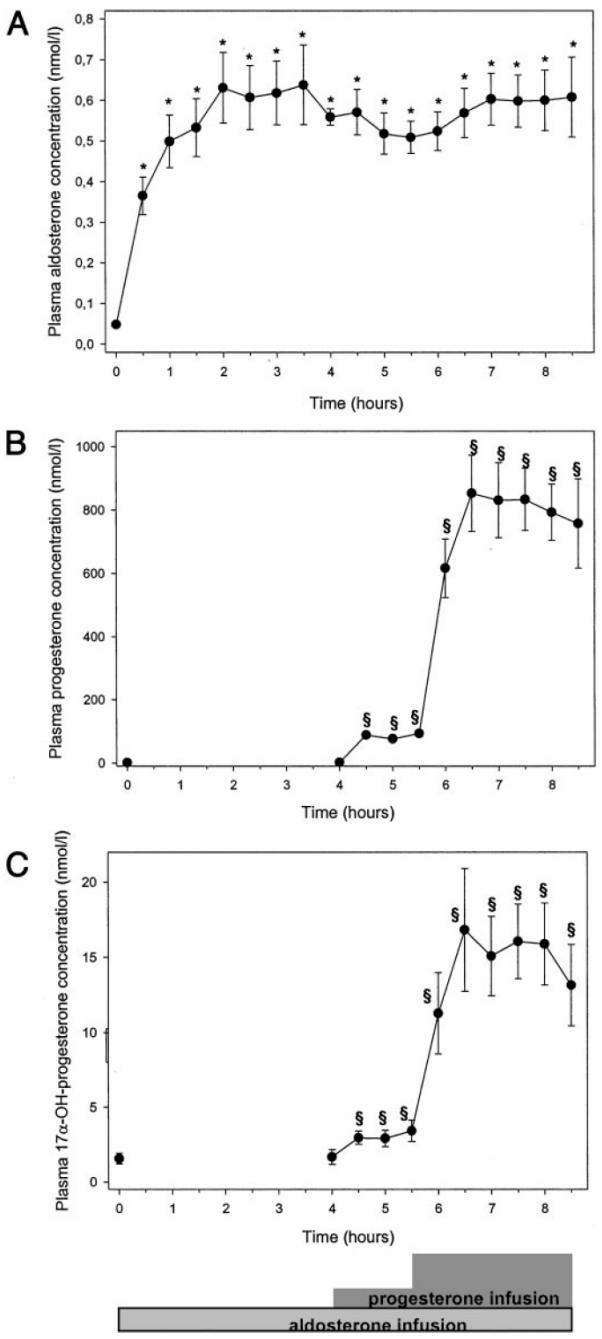

FIG. 1. Plasma aldosterone (A), P (B), and 17α-hydroxy-P (C) concentrations (mean ± SEM) during the administration of continuous aldosterone infusion over 8.5 h in eight patients without functioning adrenal glands. After 4 h a continuous progesterone infusion was started with two concentration steps. *, $P < 0.05$ compared with the starting point of aldosterone infusion (0 h). §, $P < 0.05$ compared with the starting point of progesterone infusion (4 h). (To convert P, aldosterone, and 17α-hydroxy-P concentrations to nanograms per milliliter, divide nanomoles per liter by 3.18, 2.77, and 3.03, respectively.)

nary volume (Fig. 2C), and urinary excretion of 17α-hydroxy-P (Fig. 2D) increased significantly (all $P < 0.05$) during P infusion. Urinary chloride excretion at 8.5 h did not reach a significant difference, because there were only five samples. No changes in systolic or diastolic blood pressure, serum sodium, serum potassium, or plasma renin concentrations were observed (data not shown).

At baseline, serum 4-dione concentrations were higher in males than in postmenopausal females (Fig. 3A) due to testicular androgen synthesis. However, serum 4-dione levels in both male and female patients increased significantly ($P < 0.05$; Fig. 3A).

Interestingly, serum DOC concentrations also increased significantly ($P < 0.05$) during P infusion (Fig. 3B), suggesting extraadrenal 21-hydroxylation. The serum DOC concentrations exceeded by far those during the luteal phase and normal pregnancy [0.3 nmol/liter (0.1 ng/ml) and 1.8 nmol/liter (0.6 ng/ml), respectively]. Serum concentrations of DHEA, estrone, and testosterone remained unchanged over the entire period of the study (data not shown).

To prevent hypocortisolemic crisis the subjects received prednisolone substitution on the test day. To evaluate 11β-HSD type 2 activity in the human kidney, we analyzed the urinary prednisolone to prednisone ratio. We were able to investigate only six of eight urinary samples due to interference with the HPLC measurements. The urinary prednisolone to prednisone ratio increased significantly during P infusion (Fig. 4), indicating inhibition of 11β-HSD type 2 in the kidney by P infusions.

Discussion

Male and postmenopausal patients without functioning adrenal glands seem to be ideal subjects to study the effect of P on the MR *in vivo*, due to lack of aldosterone synthesis or major gonadal P production. In our study P infusions resulted in plasma P concentrations similar to levels during the third trimester of pregnancy. Renal sodium excretion increased significantly during P infusion, resulting in a significantly increased sodium to potassium ratio. Fronius *et al.* (14) described fast, nongenomic effects of P on sodium absorption in *Xenopus* kidney. In our study P did not show a rapid nongenomic effect on sodium absorption. The increases in the urinary sodium to potassium ratio and sodium excretion after 3 h of P infusion indicates a probable gene-mediated antimineralocorticoid effect of P via the MR, *e.g.* reducing the number and expression of transport proteins, such as ENaC (amiloride-sensitive epithelial sodium channel) and Na^+/K^+-adenosine triphosphatase (15). Surprisingly, the effect was relatively small considering the 1000-fold excess of P over aldosterone, supporting the hypothesis of MR protection via renal metabolism of P (6).

A natriuretic effect of P in humans was described over 40 yr ago (16–21). In most of these studies experimental subjects received large amounts of P im over several days, and urinary sodium and aldosterone excretion was measured, but plasma concentrations of P were not determined. Therefore, the exact *in vivo* anti-MR potency of P is still not known.

The observed increase in urinary chloride excretion during P infusion in our patients is in accordance with earlier reports

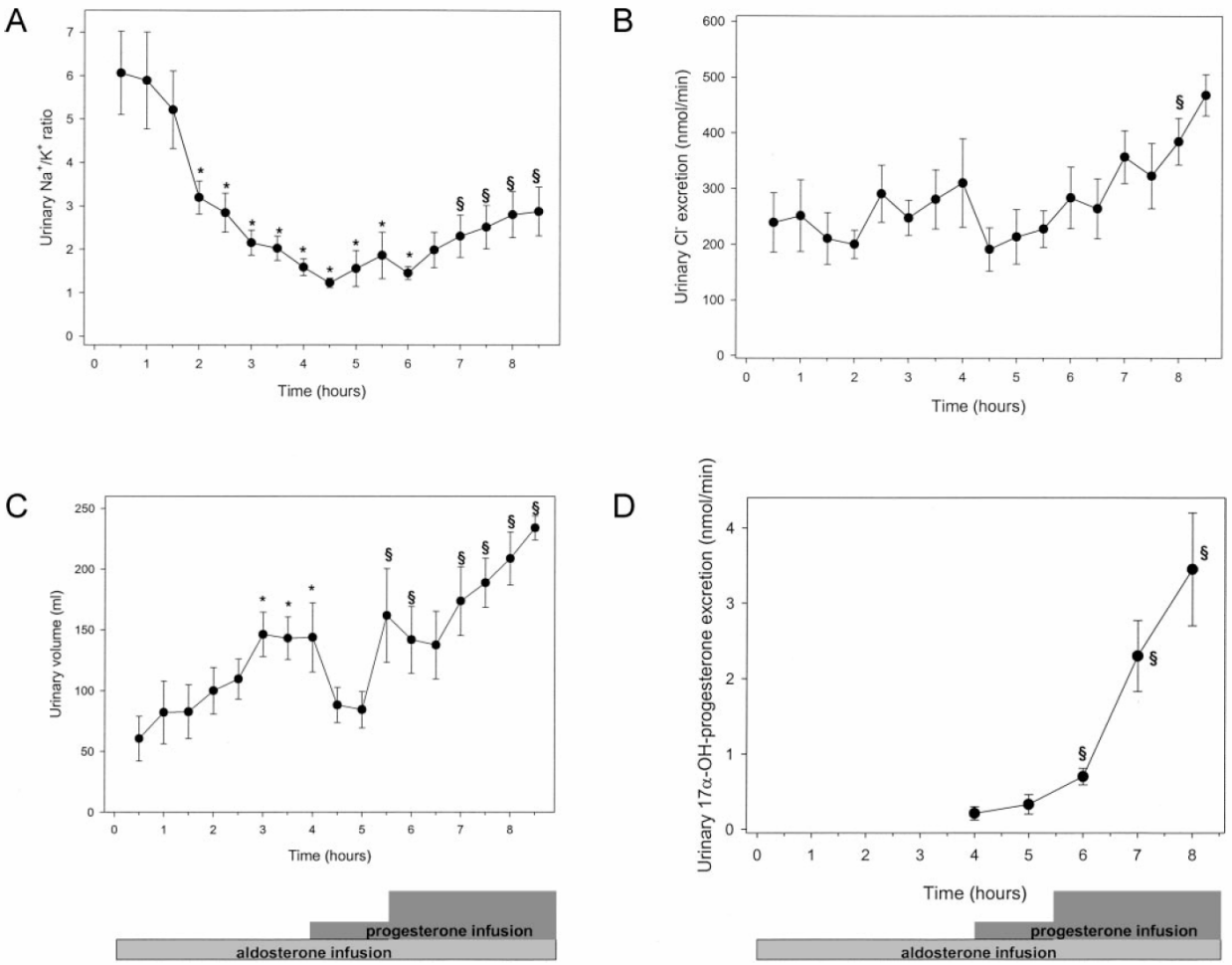

FIG. 2. Urinary sodium (Na^+) to potassium (K^+) ratio (A), urinary chloride (Cl^-) excretion (B), urinary volume (C), and urinary 17α-hydroxy-P excretion (D; mean ± SEM) during the administration of continuous aldosterone infusion over 8.5 h in eight patients without functioning adrenal glands. After 4 h a continuous progesterone infusion was started with two concentration steps. *, $P < 0.05$ compared with 0.5 h. §, $P < 0.05$ compared with 4.5 h.

(18) and could be caused by an additional inhibition of proximal sodium and chloride reabsorption in the thick ascending limb of the loop of Henle (22).

Urinary volume increased during aldosterone infusions and even more so under P infusions. This may be due to the rather high amount of liquid the patients consumed to guarantee sufficient urine flow. The further increase in urine flow during P infusion may have partly been caused by the diuretic potency of P. A similar phenomenon was described after i.m. P application in earlier reports (17). It is possible that this is caused by an additional inhibition of proximal sodium retention by P (22) or an increased renal blood flow caused by smooth muscle relaxation by P, resulting in a washout effect of the medulla (20). In accordance with this, decreased peripheral vascular resistance and increased renal plasma flow and glomerular filtration rate are observed during luteal phase and pregnancy (23).

The increase in plasma and urinary 17α-hydroxy-P in patients without functioning adrenals during P infusion indicates an extraadrenal conversion of P to 17α-hydroxy-P, possibly in the gonads or even in the kidney. The increased urinary 17α-hydroxy-P excretion may be based on glomerular filtration as well as on renal production, thus proposing a possible intrarenal conversion of P to 17α-hydroxy-P. 17α-Hydroxy-P is a major renal P metabolite *in vitro* and has weaker affinity and antagonistic properties than P to the MR (3, 6). In human kidney tissue, 17α-hydroxy-P is further inactivated to 17α-hydroxy-20α-dihydroprogesterone, which has very little intrinsic activity for the human MR (3, 6, 7).

In humans the main pathway for androgen synthesis is from pregnenolone via 17α-hydroxypregnenolone to DHEA (24). The pathway from P via 17α-hydroxy-P to 4-dione is energetically not preferred (25). Nevertheless, we found a significant increase in plasma 4-dione concentrations during P infusion with no change in plasma DHEA concentrations. 4-Dione may originate from the gonads, where 3β-HSD ex-

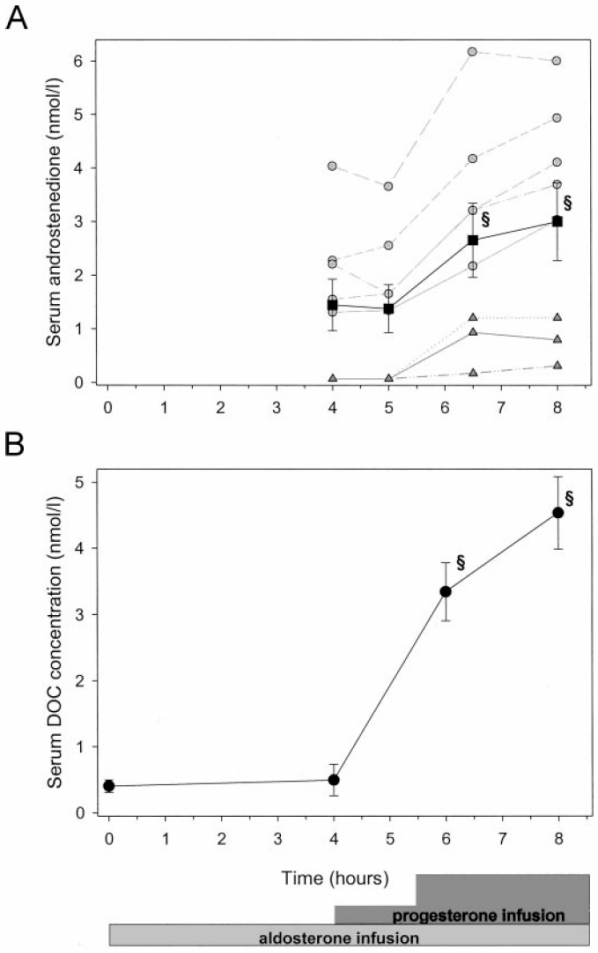

FIG. 3. Serum 4-dione (A) and DOC (B) concentrations (mean ± SEM) during the administration of continuous aldosterone infusion over 8.5 h in eight patients without functioning adrenal glands. After 4 h a continuous progesterone infusion was started with two concentration steps. §, $P < 0.05$ compared with starting point of progesterone infusion (4 h). ○, Male subjects; △, female subjects. (To convert 4-dione and DOC concentrations to nanograms per milliliter, divide nanomoles per liter by 3.49 and 3.03, respectively.)

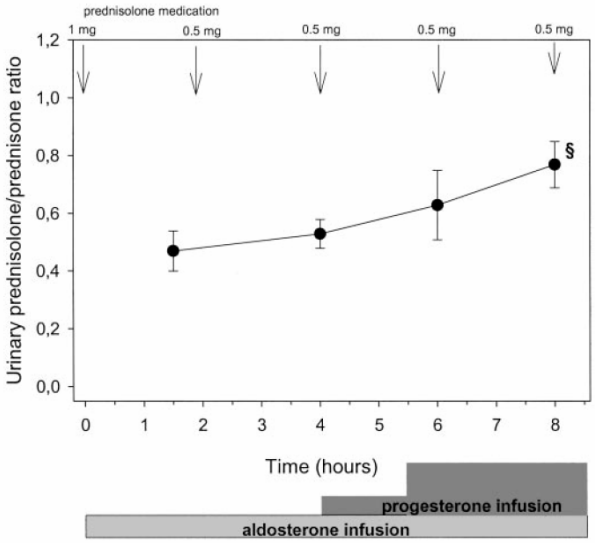

FIG. 4. Urinary prednisolone to prednisone ratio (mean ± SEM) during the administration of continuous aldosterone infusion over 8.5 h in six patients without functioning adrenal glands. After 4 h a continuous progesterone infusion was started with two concentration steps. §, $P < 0.05$ compared with starting point of progesterone infusion (4 h).

pression by far exceeds that of P450c17, resulting in the release of predominantly 4-dione rather than DHEA. Concordantly, adrenal suppression with dexamethasone leads to almost complete suppression of serum DHEA/DHEA sulfate, whereas 4-dione is only reduced to 30% (26).

We showed that plasma DOC concentrations rose significantly during P infusion and reached higher levels than during the luteal phase and pregnancy in normal women. This indicates an extraadrenal conversion of P by 21-hydroxylase to the MR agonist DOC and supports previous reports that local formation of DOC in renal tissue might be an important para- or autocrine mechanism to protect the MR (27–30).

P is a very potent inhibitor of 11β-HSD type 2 (6). Therefore, it is interesting to investigate the *in vivo* inhibition of renal 11β-HSD type 2 under high P concentrations. The urinary prednisolone to prednisone ratio increased significantly during P infusions. This indicates an inhibition of renal 11β-HSD type 2. Due to this inhibition, less cortisol is inactivated to cortisone, and therefore more endogenous cortisol can bind as agonist to the MR (6). This may be an additional mechanism for sufficient MR activation in states of high P concentrations. It is possible that the prednisolone used in the study to prevent hypocortisolemic crisis may have had some mineralocorticoid effect. We consider this unlikely, however, because of the low dose of prednisolone used and the strong inactivation to prednisone by 11β-HSD type 2. Although inhibition of 11β-HSD type 2 may have a role in the attenuated antimineralocorticoid effect of P *in vivo*, it seems more likely that the 10-fold increase in DOC concentrations is the major cause of this effect.

In conclusion, the increase in the urinary sodium to potassium ratio and urinary sodium excretion during P infusion indicated an anti-MR effect of P. However, this effect was much weaker than would be expected from the rise in circulating P concentration. This may be partly explained by an effective enzyme-mediated protection of the MR and the extraadrenal DOC synthesis, as indicated by increased serum DOC concentrations during P infusion. An additional protective mechanism could be an inhibition of 11β-HSD type 2 by P, thus giving the MR agonist cortisol access to the MR. The increases in serum and urinary 17α-hydroxy-P and serum 4-dione in these patients without functioning adrenal glands indicate extraadrenal conversion of P.

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3.2.3 Identifizierung der Progesteron metabolisierenden Enzyme

10. Bumke-Vogt C, Bähr V, Diederich S, Herrmann SM, Agnostopoulos I, Oelkers W, **Quinkler M.**
    Expression of the progesterone receptor and progesterone-metabolising enzymes in the female and male human kidney.
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Expression of the progesterone receptor and progesterone-metabolising enzymes in the female and male human kidney

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Abstract

Due to high binding affinity of progesterone to the human mineralocorticoid receptor (hMR), progesterone competes with the natural ligand aldosterone. In order to analyse how homeostasis can be maintained by mineralocorticoid function of aldosterone at the MR, especially in the presence of elevated progesterone concentrations during the luteal phase and pregnancy, we investigated protective mechanisms such as the decrease of free progesterone by additional binding sites and progesterone metabolism in renal cells. As a prerequisite for sequestration of progesterone by binding to the human progesterone receptor (hPR) we demonstrated the existence of hPR expression in female and male kidney cortex and medulla at the level of transcription and translation. We identified hPR RNA by sequencing the RT-PCR product and characterised the receptor by ligand binding and Scatchard plot analysis. The localisation of renal hPR was shown predominantly in individual epithelial cells of distal tubules by immunohistology, and the isoform hPR-B was detected by Western blot analysis. As a precondition for renal progesterone metabolism, we investigated the expression of steroid-metabolising enzymes for conversion of progesterone to metabolites with lower affinity to the hMR. We identified the enzyme 17α-hydroxylase for renal 17α-hydroxylation of progesterone. For 20α-reduction, different hydroxysteroid dehydrogenases (HSDs) such as 20α-HSD, 17β-HSD type 5 (3α-HSD type 2) and 3α-HSD type 3 were found. Further, we detected the expression of 3β-HSD type 2 for 3β-reduction, 5α-reductase (Red) type 1 for 5α-reduction, and 5β-Red for 5β-reduction of progesterone in the human kidney. Therefore metabolism of progesterone and/or binding to hPR could reduce competition with aldosterone at the MR and enable the mineralocorticoid function.

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Introduction

Progesterone is one of the main steroid hormones involved in the regulation of female reproductive function (Graham & Clarke 1997). Its effects are mediated by the progesterone receptor (PR), a member of the nuclear receptor family of ligand-activated transcription factors (Tsai & O'Malley 1994). Upon binding of progesterone to the ligand-binding domain (Williams & Sigler 1998), the whole receptor protein undergoes conformational changes leading to dissociation from heat shock protein (hsp) such as hsp90 (Pratt & Toft 1997). This enables dimerisation of receptor monomers and binding to hormone-responsive elements of DNA within the regulatory region of target genes (Beato *et al.* 1987). Transcription or suppression of the target gene depends on the context of promoter and the distribution of PR isoforms in target cells. Two isoforms of the PR have been described (Horwitz & Alexander 1983): PR-B (933 amino acids) and PR-A (769 amino acids), the latter lacking 164 N-terminal amino acids of PR-B. Both isoforms are expressed from the same gene by transcription from two alternative promoters and translation from two different start codons located in the transcript of the first exon (Kastner *et al.* 1990*a*). PR expression has been described in classical target organs like the uterus (Bergeron *et al.* 1988), ovary (Duffy & Stouffer 1995), vagina (Batra & Iosif 1985), breast (Horwitz & McGuire 1975) and brain (pituitary gland and hypothalamus) (Kato *et al.* 1978). In endometrial stromal cells, progestins induce target genes encoding transforming growth factor-β and insulin-like growth factor-binding protein (IGFBP)-1. The IGFBP-1 promoter is more

strongly induced by PR-A than by PR-B (Gao *et al.* 2000). In uterine epithelia, the stimulating effect of progesterone on the expression of histidine decarboxylase is mediated by PR-B, as shown by experiments with knockout mice for PR-A, while PR-A seems to be essential for a progesterone activation of amphiregulin and calcitonin expression (Mulac-Jericevic *et al.* 2000). In the breast cancer cell line T-47D, progestins induce the expression of desmoplakin, CD59/protectin, FKPB51, and the Na^+/K^+-ATPase subunit α1. The latter is also found in normal breast tissue (Kester *et al.* 1997). In T-47D, expression of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 is increased by progestins (Arcuri *et al.* 2000). For 11β-HSD type 2, co-localisation with the mineralocorticoid receptor (MR) has been demonstrated in normal and malignant human breast tissue (Sasano *et al.* 1997), where it could facilitate selective binding of aldosterone to MR, as has been shown in the kidney (Edwards *et al.* 1988). McDonnell *et al.* (1994) described the possibility of a repression of MR transcriptional activity by ligand-activated PR-A via cross-talk of MR with this potent transdominant inhibitor competing for a common transcription factor or 'adaptor'.

The question whether the PR is also expressed in the human kidney is of special interest, since progesterone also binds with high affinity to the renal MR but confers only weak transcriptional activity. Hence progesterone is a strong MR antagonist (Rupprecht *et al.* 1993*a*, Myles & Funder 1996). Geller *et al.* (2000) have described an agonistic progesterone function at the mutated MR_{L810}, found in a family of patients with early onset of severe hypertension. Pregnant members developed preeclampsia caused by a normal increase of progesterone. To determine how homeostasis is maintained in late pregnancy, for example, when the plasma progesterone concentration can rise to 700 nM (Johansson & Jonasson 1971) with an aldosterone increase to 5·8 nM (Nolten *et al.* 1978), we examined two mechanisms for avoiding excessive progesterone binding at the MR.

One explanation for the discrepancy of high progesterone concentration in the presence of wild-type MR and a still functioning renin–aldosterone system (Oelkers 1996) could be a competition of PR with MR for binding of progesterone in the kidney. Therefore we examined the expression of the human (h)PR in renal cortex and medulla of female (pre- and postmenopausal) and male origin. The possibility of high-specificity binding of progesterone to hPR in mineralocorticoid target cells could prevent antagonism or agonism of progesterone at the MR in the kidney.

Another mechanism for reducing progesterone binding to MR is the metabolic conversion of progesterone to derivatives with lower affinity to hMR. In renal cell fractions an effective metabolism of progesterone has been described as: 17α-OH(hydroxy)-progesterone(P) (23–32%), 20α-DH(dehydro)-P (24–27%), 17α-OH,20α-DH-P (9–11%), 5α-DH-P (7–8%), 20α-DH,5α-DH-P (5%), 3β,5α-TH(tetrahydro)-P (2–3%), 20α-DH,3β,5α-TH-P (2%), 3α,5α-TH-P or 3β,5β-TH-P (1–2%), and 5β-DH-P (<1%) (total conversion of more than half of progesterone in the range of 1 nM–1 μM) (Quinkler *et al.* 1999, 2001). These metabolites exhibit reduced affinity to the MR in comparison with progesterone: 7% for 17α-OH-P, 11% for 20α-DH-P, 0·2% for 17α-OH,20α-DH-P, 9% for 5α-DH-P, 0·4% for 20α-DH,5α-DH-P, 0·5% for 3β,5α-TH-P,<0·1% for 20α-DH,3β,5α-TH-P,<0·1% for 3α,5α-TH-P,<0·1% for 3β,5β-TH-P, and 0·6% for 5β-DH-P (Quinkler *et al.* 2002). Thus the following steroid-metabolising enzymes were examined for renal expression in female (premenopausal) and male kidney cortex and medulla: for 17α-hydroxylation, 17α-hydroxylase/17,20-lyase (P450c17 or CYP17) (Chung *et al.* 1987), for 20α-reduction, 20α-HSD (aldo-keto reductase AKR1C1) (Nishizawa *et al.* 2000), 3α-HSD type 3 (AKR1C2) (Shiraishi *et al.* 1998), prostaglandin F synthase (PGFS, AKR1C3) (Suzuki-Yamamoto *et al.* 1999), and 17β-HSD type 5 (Dufort *et al.* 1999) with 20α-HSD activity, for 5α-reduction, both isoforms 5α-reductase (Red) type 1 and type 2 (Andersson & Russell 1990, Andersson *et al.* 1991), for 5β-reduction, 5β-Red (Kondo *et al.* 1994), and for 3β-reduction, both isoforms 3β-HSD type 1 and type 2 (Rheaume *et al.* 1991, Dumont *et al.* 1992).

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CV-1 and MCF7 cells were cultured in Dulbecco's MEM (modified Eagle's medium) supplemented with 10% fetal calf serum (FCS) and 100 000 IU/l penicillin and 100 mg/l streptomycin. For culture of CV-1, 2·5 mg/l amphotericin B and for MCF7, 2 mM glutamine were added. T-47D were maintained in RPMI 1640 medium with 10% FCS, 100 000 U/l penicillin and 100 mg/l streptomycin, 1 mM sodium-pyruvate and

strongly induced by PR-A than by PR-B (Gao *et al.* 2000). In uterine epithelia, the stimulating effect of progesterone on the expression of histidine decarboxylase is mediated by PR-B, as shown by experiments with knockout mice for PR-A, while PR-A seems to be essential for a progesterone activation of amphiregulin and calcitonin expression (Mulac-Jericevic *et al.* 2000). In the breast cancer cell line T-47D, progestins induce the expression of desmoplakin, CD59/protectin, FKPB51, and the Na^+/K^+-ATPase subunit α1. The latter is also found in normal breast tissue (Kester *et al.* 1997). In T-47D, expression of the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 is increased by progestins (Arcuri *et al.* 2000). For 11β-HSD type 2, co-localisation with the mineralocorticoid receptor (MR) has been demonstrated in normal and malignant human breast tissue (Sasano *et al.* 1997), where it could facilitate selective binding of aldosterone to MR, as has been shown in the kidney (Edwards *et al.* 1988). McDonnell *et al.* (1994) described the possibility of a repression of MR transcriptional activity by ligand-activated PR-A via cross-talk of MR with this potent transdominant inhibitor competing for a common transcription factor or 'adaptor'.

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(1 min, 10 000 ***g***) and each pellet washed twice with 500 µl buffer A. Following resuspension in 20 µl 2 × SDS loading buffer containing 215 mM Tris–HCl pH 6·7, 17·4% glycerol, 5·2% SDS, 8·7% β-mercaptoethanol and 0·27% bromphenol blue, samples were denatured at 97 °C for 5 min. Protein A-Sepharose was pelleted by centrifugation (1 min, 10 000 ***g***) and the supernatant used for protein analysis.

SDS-PAGE, Western transfer blotting and immunodetection Polyacrylamide mini-gels for protein electrophoresis were prepared as described in the manual (Hoefer, San Francisco, CA, USA) with two layers of 8 and 5% polyacrylamide gels containing 0·1% SDS respectively. Protein samples in SDS loading buffer and a prestained protein ladder (Bio-Rad) were separated during electrophoresis with 20 mA/gel for 1 h and proteins were blotted by electrotransfer with 400 mA for 2 h to PVDF membranes (Bio-Rad). Membranes were incubated overnight in a blocking solution containing 0·5% casein and 0·1% Tween-20 in PBS (Vector Laboratories, Burlingame, CA, USA). Blocked membranes were incubated for 1 h at room temperature (RT) with primary antibody NCL-PGR 312 (mouse monoclonal anti-hPR (PR-A and PR-B) IgG) (Novocastra, Newcastle, UK) diluted 1:1000 in blocking solution and, following extensive washing, for 1 h at RT with the secondary antibody goat anti-mouse IgG conjugated to peroxidase (Sigma, Deisenhofen, Germany), diluted 1:4000 in blocking solution. Chemiluminescence of the peroxidase reaction was detected by ECL or ECL Plus (Amersham Pharmacia Biotech). PVDF membranes were stripped for 30 min at 50 °C in 62·5 mM Tris–HCl pH 6·7, 100 mM β-mercaptoethanol, 2% SDS, blocked and reincubated with NCL-PGR 312, followed by a different secondary antibody (biotinylated anti-mouse IgG diluted 1:300) and horseradish peroxidase-streptavidin diluted 1:500 (Vector).

Preparation of RNA for gene expression analysis

RNAs from tissues (150–250 mg snap-frozen kidney cortex and medulla specimens) and from cultured cells (10^6-10^7 cells of MCF7 or CV-1) were isolated immediately after thawing of tissues or directly after harvesting of cells respectively with the RNeasy Kit (Qiagen, Hilden, Germany) including an on-column DNase digestion with the RNase-free DNase set also provided by Qiagen. The concentration and quality of RNA was determined photometrically (260/280 nm).

RT-PCR

RNA (2·5 µg) was annealed with random hexamer primers, 15 µg/45 µl incubation volume, for 10 min at 70 °C. First-strand reverse transcription was performed in 100 µl mixtures with 1000 U RT II Superscript polymerase in the presence of 40 U RNase inhibitor, 1 µmol DTT, 50 nmol dATP, 50 nmol dGTP, 50 nmol dCTP, 50 nmol dTTP in first-strand buffer for 1 h at 37 °C followed by denaturation for 5 min at 95 °C. All supplements were obtained from Gibco BRL (Karlsruhe, Germany). Specific PCRs with synthesised cDNA templates (transcribed from 40 ng RNA) were carried out in amplification mixtures of 25 µl containing 20 mM Tris–HCl pH 8·4, 50 mM KCl, 1·5 mM $MgCl_2$, and 0·2 mM each of dATP, dCTP, dGTP, dTTP using 1 U Taq DNA polymerase (Gibco BRL) and 12 pmol specific sense and antisense primers (Tib Molbiol, Berlin, Germany) listed in Table 1.

PCRs were performed with an initial step of denaturation for 2 min at 94 °C and were run for 35 cycles with denaturation for 45 s at 94 °C, annealing for 1 min at the thermodynamic melting temperature of the specific primer pair (Table 1), and extension for 90 s at 72 °C followed by a final extension for 10 min at 68 °C. Amplicons were resolved by electrophoresis in 1% agarose gels stained with ethidium bromide. The size and quantity of PCR products were approximated by comparison with a 1 kb Plus-DNA ladder and DNA low-mass ladder (Gibco BRL). PCR products of expected size were re-extracted from agarose gels (QIAquic gel extraction kit; Qiagen) for direct sequencing. Each sample was sequenced twice with the specific sense and antisense primers (Table 1) respectively using an automated sequencing device (ABI Prism 377; Perkin-Elmer, Überlingen, Germany), as described previously by Herrmann *et al.* (2001). Sequences were identified by blast search GenBank analysis.

hPR-specific high-stringency PCR

The conditions of hPR-specific RT-PCR were similar to those described (Misao *et al.* 1998) with 35 instead of 25 cycles using an annealing temperature of 55 °C for the primer pair hPR sense and hPR antisense (Table 1). We investigated cDNAs from tissue specimens of kidney cortex and medulla of a premenopausal, a postmenopausal, and a male patient. The PR-expressing breast carcinoma cell line MCF7 was used as positive control. The vector pRShMR which contains the complete coding sequence (CDS) for the hMR (Arriza *et al.* 1987), kindly provided by R Evans, was used as negative control for the hPR-specific PCR.

High-stringency PCRs specific for progesterone-metabolising enzymes

Specific primers were designed according to published sequences of steroid-converting enzymes and PCRs were performed with 35 cycles using primer pair-specific annealing temperatures as summarised in Table 1.

Table 1 Gene-specific primers for high-stringency PCRs. Sets of gene-specific primer pairs (s=sense, as=antisense) for amplification of cDNA according to the published CDS (first reference) and genomic location according to the exons of the published gene sequences (second reference) of the hPR and progesterone-metabolising enzymes are shown. Calculated sizes of the expected amplicon and the primer pair specific annealing temperatures are indicated for PCRs (2 min 94 °C, 35 cycles: 45 s 94 °C, 1 min × °C, 90 s 72 °C, and 10 min 68 °C). Note that 17β-HSD type 5 specific primers were designed to the high homologous sequence of 3α-HSD (clone HAKRb) because no sequence for 17β-HSD type 5 was available in the GeneBank

	Primer (sense); primer (antisense)	Genomic location	Annealing temperature (°C)	Expected size of amplified cDNA (bp)	According to published sequences (references)
Specific primers for hPR and enzymes					
hPR s	5′-AGCCCTAAGCCAGAGATTCA-3′	Exon 4	55	303	Misrahi *et al.* (1987)
hPR as	5′-TAGGATCTCCATCCTAGACC-3′	Exon 5			Misrahi *et al.* (1993)
17α-hydroxylase s	5′-TCTCTTGCTGCTTACCCTAG-3′	Exon 1	55	527	Chung *et al.* (1987)
17α-hydroxylase as	5′-TCAAGGAGATGACATTGGTT-3′	Exon 3			Picado Leonard & Miller (1987)
20α-HSD s	5′-GGGATCCCACCGAGAAGAACC-3′	Exon 6	65	416	Stolz *et al.* (1993)
20α-HSD as	5′-TAACCAGAGGCGATGTGTCCAGTC-3′	Exon 9			Lou *et al.* (1994)
3α-HSD type 3 s	5′-TAAAAGTAAAGCTCTAGAGGCCGT-3′	Exon 2	55	191	Ciaccio & Tew (1994)
3α-HSD type 3 as	5′-ACTCTGGTCGATGGGAATTGCT-3′	Exon 3			Khanna *et al.* (1995)
PGFS s	5′-ATTCCGGGCAGCAGCAAACA-3′	Exon 1	55	562	Suzuki-Yamamoto *et al.* (1999)
17β-HSD type 5 as1*	5′-CTGCCTGCGGTTGAAGTTTGATA-3′	Exon 5			Khanna *et al.* (1995)
17β-HSD type 5 s1	5′-ACTTCATGCCTGTATTGGGATTTG-3′	Exon 1	60	476	Qin *et al.* (1993)
17β-HSD type 5 as1*	5′-CTGCCTGCGGTTGAAGTTTGATA-3′	Exon 5			Khanna *et al.* (1995)
17β-HSD type 5 s2	5′-GGAGGCCATGGAGGAGTGTAAGGA-3′	Exon 4	65	335	Qin *et al.* (1993)
17β-HSD type 5 as2	5′-GGTAGCGCAGGGCAATCAGG-3′	Exon 7			Khanna *et al.* (1995)
17β-HSD type 5 s3	5′-AACGAGACAAACGATGGGTGGAC-3′	Exon 6	60	469	Qin *et al.* (1993)
17β-HSD type 5 as3	5′-GTATTTCTGGCCTATGGAGTGAGC-3′	Exon 9			Khanna *et al.* (1995)
3α-HSD s	5′-GTGACAGGGAATG-3′	Exon 1	40	248	Qin *et al.* (1993)
3α-HSD as	5′-ATATGTCTTCTCTCTCA-3′	Exon 2			Khanna *et al.* (1995)
3β-HSD type 1 s	5′-GATCATCCGCCTCCTGGTG-3′	Exon 2	55	485	Dumont *et al.* (1992)
3β-HSD type 1 as	5′-GGGTGCCGCCGTTTTTCA-3′	Exon 4			Lachance *et al.* (1990)
3β-HSD type 2 s	5′-GATCGTCCGCCTGTTGGTG-3′	Exon 2	65	382	Rheaume *et al.* (1991)
3β-HSD type 2 as	5′-CTCTTCTTCGTGGCCGTTCTGGATGAT-3′	Exon 4			Lachance *et al.* (1991)
5α-Red type 2 s	5′-GCCCGGCAGCCCCTCTCC-3′	Exon 1	60	244	Andersson *et al.* (1991)
5α-Red type 2 as	5′-CACCCAAGCTAAACCGTATGTCTG-3′	Exon 2/3			Labrie *et al.* (1992)
5α-Red type 1 s	5′-CGGGCCGCCTGGGTGGTG-3′	Exon 1	65	168 (gene)	Andersson & Russell (1990)
5α-Red type 1 as	5′-TCCTCGCATCAGAAACGGGTAAAT-3′	Exon 2		168 (pseudo)	Jenkins *et al.* (1991)
5α-Red type 1 preATG s	5′-CTGGGGCATGGAGCACGC-3′	Exon 1	65	351 (gene)	Andersson & Russell (1990)
5α-Red type 1 as	5′-TCCTCGCATCAGAAACGGGTAAAT-3′	Exon 2		357 (pseudo)	Jenkins *et al.* (1991)
5α-Red type 1 pseudo s	5′-GGCCATGTTCCTCGTCCACTAC-3′	Exon 1	60	—	—
5α-Red type 1 pseudo as	5′-CCGCTCATGATGCTCTTTTGCTCT-3′	Exon 1		454 (pseudo)	Jenkins *et al.* (1991)
5α-Red type 1 gene s	5′-CAGATCCCCGTTTTCTAATAGG-3′	Exon 2	55	239 (gene)	Andersson & Russell (1990)
5α-Red type 1 gene as	5′-AAACGTGAAGAAAGCAAAAGC-3′	Exon 4			Jenkins *et al.* (1991)
5β-Red s	5′-AACAAGCCAGGACTCAAACACAAG-3′	Exon 5	60	451 (gene)	Kondo *et al.* (1994)
5β-Red as	5′-CTCCCGGCAGTCAGTATTCATCA-3′	Exon 9		432 (pseudo)	Charbonneau & The (2001)

*The antisense primer (17β-HSD type 5 as1) was also used for PGFS-specific cDNA amplification as there is only one mismatch in position 22. preATG, 5′ to start codon ATG; pseudo, pseudogene.

Table 2 Comparison of ligand binding characteristics. Scatchard plot analyses were performed with [^{3}H] R5020 using steroid-depleted cytosol fractions from tissue specimens of human kidney cortex and medulla (postmenopausal), myometrium (postmenopausal) as classical target tissue control and from hPR-expressing breast carcinoma cells MCF7 and T-47D

	K_d (nM)	B_{max} (fmol/mg protein)
Tissues and cells		
Kidney cortex	0·76	7
Kidney medulla	0·86	17
Myometrium	0·98	213
MCF7	0·57	43
T-47D	0·44	347

K_d=dissociation constant for specific binding of promegestone (R5020) to the receptor (hPR); B_{max}=maximum concentration of available binding sites (calculated using the linear regression method).

Templates for enzyme expression analyses were cDNAs from female (premenopausal) and male kidney samples and from CV-1 monkey kidney cells as control. Additionally cDNA templates from human adrenals and testes, kindly provided by W Arlt, were used as positive controls for steroid-metabolising enzyme expression.

Results

Characterisation of the hPR by binding of promegestone (R5020)

Scatchard plot analysis was performed using steroid depleted cytosol fractions from renal cortex and medulla as well as from myometrium (target tissue control). Additional positive controls were MCF7 and T-47D breast carcinoma cells, the latter overexpressing hPR-A. Specific binding of promegestone (R5020) as a highly specific ligand for the PR was found in kidney cortex and medulla specimens as well as in all controls. The binding affinities, characterised by their dissociation constant (K_d values) calculated by linear regression analysis, were in the same range (0·44–0·98 nM) for all samples (Table 2). Therefore the existence of the same kind of receptor in both kidney specimens and the progesterone target tissue myometrium as well as in hPR-expressing cells could be assumed. The amount of R5020-specific binding protein (B_{max}) in the kidney (7–17 fmol/mg protein) was less than 10% compared with the target tissue myometrium (213 fmol/mg protein). B_{max} in MCF7 cells was 43 fmol/mg protein and in T-47D cells 347 fmol/mg protein (Table 2).

Localisation of hPR in human kidneys by immunohistology

In tissue sections of female postmenopausal (Fig. 1A and B), premenopausal (Fig. 1C and D) and male (Fig. 1F) human kidneys and of myometrium (Fig. 1E) used as positive control, hPR was detected as nuclear staining following treatment with the antibody PgR 636, APAAP and Fast Red chromogen. A high percentage of myometrial smooth muscle cells stained positively for hPR (Fig. 1E). Labelling for hPR was also detected in kidney specimens. Positive immunodetections of hPR were found predominantly in some individual epithelial cells of distal tubules in female (Fig. 1A–C) and male (Fig. 1F), in single podocytes of glomeruli in postmenopausal (Fig. 1B), premenopausal (Fig. 1D), and male (Fig. 1F), in parietal cells in premenopausal (Fig. 1D) and in few interstitial cells of investigated renal tissues. The cytoplasmic staining (Fig. 1D and F) is probably not PR-specific but artefactual (reaction of anti-alkaline phosphatase, used in APAAP, with the renal alkaline phosphatase).

hPR-B is synthesised in female and male kidneys

Western blots of protein fractions prepared by immunoprecipitation of hPR gave chemiluminescent signals in human male and female kidney samples as a single band, corresponding to the middle band of the triplet for PR-B in cytoplasm of T-47D used as control (114, 117, 120 kDa) (Sheridan *et al.* 1989). This 117 kDa protein band was hardly detectable in a sample of cytosol from male kidney cortex with 200 μg protein content without immunoprecipitation of hPR, but the immunoprecipitates from cytosol aliquots with 5000, 1000 and 500 μg protein gave clear concentration-dependent chemiluminescent signals (Fig. 2A). This dependence on protein concentration used for immunoprecipitation was also found in total protein extracts from female kidney cortex and medulla (Fig. 2B). Smears of chemiluminescent signals in the range of slightly higher to lower size of PR-A (detectable in all samples) could not be identified as PR-A as in T-47D. After membrane stripping and reincubation with the identical first antibody, NCL-PGR 312, and a different second antibody (biotinylated anti-mouse IgG followed by horseradish peroxidase-streptavidin detection), chemiluminescence did not appear directly at the position of PR-A (compared with T-47D), but there were strong signals at the position of PR-B, besides non-specific bands smaller than PR-B and PR-A (Fig. 2A and B, lower panel). Therefore, only the synthesis of PR-B could be clearly shown in male and female kidney specimens.

hPR-gene expression in the kidney

The transcription of the hPR-gene analysed by RT-PCR was found in all of our kidney samples (Fig. 3). From cDNAs of renal cortex and medulla from premenopausal, postmenopausal and male patients, PCR products of 303 bp were amplified with the hPR-specific primer pair (Table 1), similar to the positive control MCF7 (PR-expressing cell line). The negative control did not give any DNA staining with ethidium bromide. In

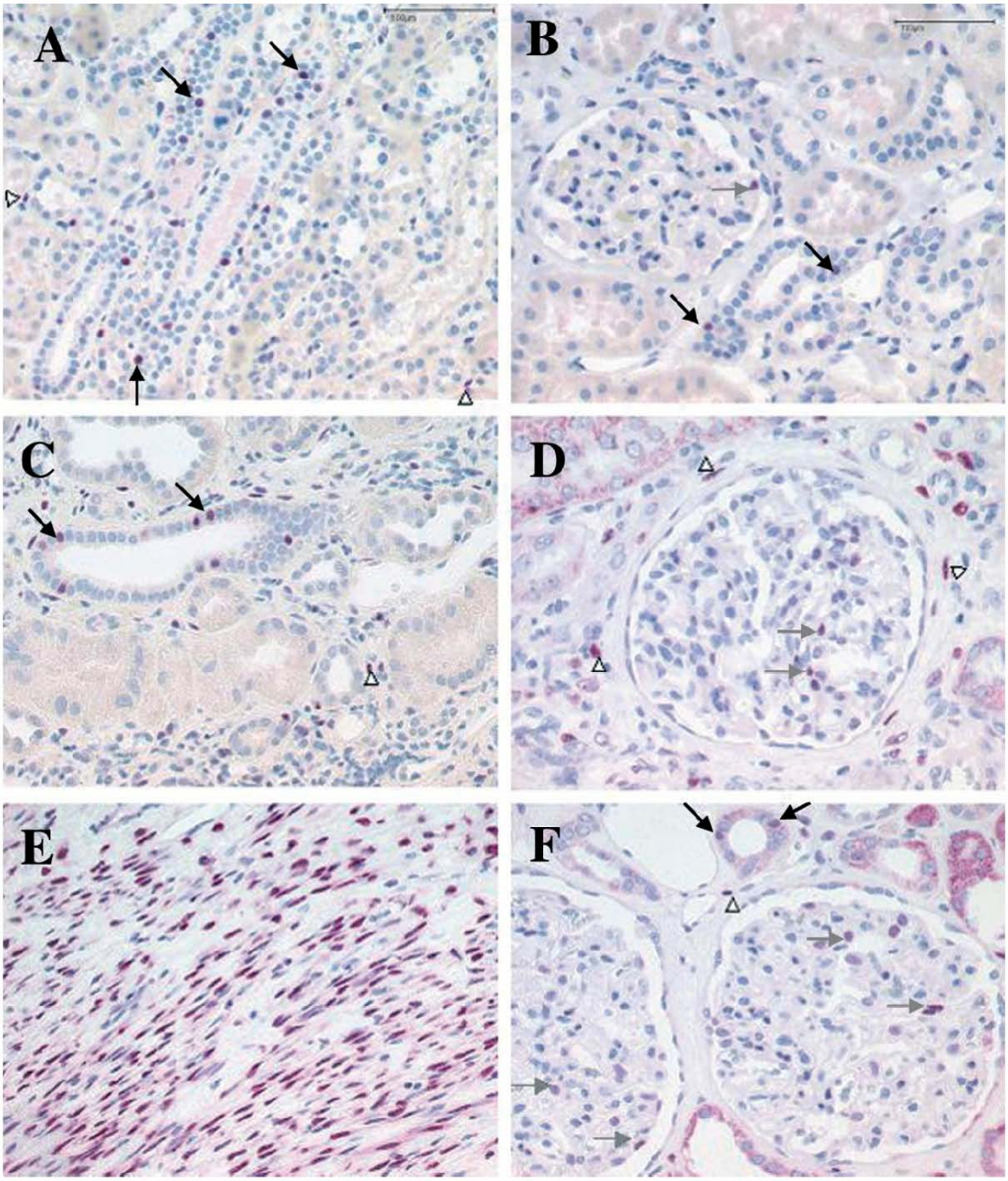

Figure 1 Immunohistological demonstration of PR expression in human kidney tissues. Immunohistological visualisation of hPR was performed on paraffin-embedded renal sections (4 µm) using PgR 636 (1:100) and APAAP complex formation with Fast Red chromogen. Demonstration of PR-positive cells in the epithelia of distal tubules (black arrows), glomeruli (grey arrows), and interstitium (white arrow-heads) in kidneys of different origin: postmenopausal (A, B), premenopausal (C, D), male (F) human kidney. Control staining for hPR was performed on myometrium (E).

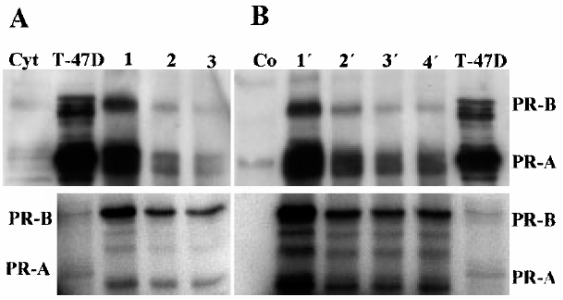

Figure 2 Western blot and immunodetection with NCL-PGR 312. (A) SDS-PAGE of 200 µg total protein from cytosol (Cyt) of male kidney cortex without immunoprecipitation, of 10 µg total protein from T-47D cells overexpressing PR-A as positive control, and immunoprecipitates obtained with PR(C20) sc539 rabbit polyclonal IgG from 5000 µg (lane 1), 1000 µg (lane 2), 500 µg (lane 3) protein in cytosol. (B) SDS-PAGE of PR(C20) sc539 under conditions of immunoprecipitation without renal proteins as negative control (Co), immunoprecipitates from 5000 µg (lane 1′), 1000 µg (lane 2′), 500 µg (lane 3′) total protein extract (cytosolic and nuclear proteins) of female kidney cortex and from 500 µg (lane 4′) of female kidney medulla, and 10 µg T-47D protein containing PR-A and PR-B as positive control. (A, B) Proteins were transferred to PVDF membranes and hPR immunodetected with NCL-PGR 312 mouse monoclonal anti-hPR IgG (1:1000) and goat anti-mouse IgG conjugated to peroxidase (1:4000) and visualised by chemiluminescence in the upper panel. There are concentration-dependent specific chemiluminescent signals as a single band at the position of PR-B in all kidney samples corresponding to the middle band in the triplet of PR-B (117 kDa) visible in T-47D, which was not detected in the negative control. A smear of chemiluminescence around the expected size of PR-A was found in all samples, but without concentration at the position of the PR-A-specific band as in the track of T-47D. There was a faint signal in the negative control evoked by the immunoprecipitating antibody itself as well. In the lower panel only PR-B could be detected after stripping of the membranes (A) and (B) and immunodetection with NCL-PGR 312 as well followed by biotinylated anti-mouse IgG and horseradish peroxidase-streptavidin. Chemiluminescent signals at positions smaller than PR-B and PR-A seem to be non-specific for PR.

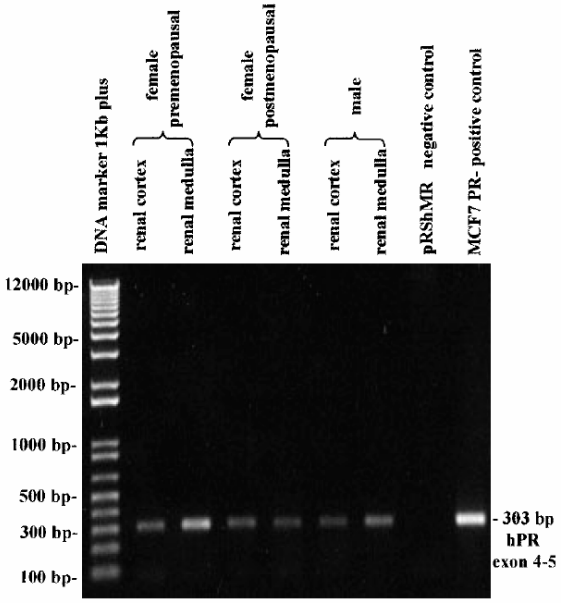

Figure 3 RT-PCR analysis for the expression of hPR. cDNAs obtained by reverse transcription of RNA from kidney cortex and medulla specimens of female (pre- and postmenopausal) and male origin and from MCF7 cells expressing the hPR as positive control were amplified by high-stringency hPR-specific PCR. pRShMR was used as template for the negative control. PCR products were separated on a 1% agarose gel with a 1 kb-Plus DNA ladder for sizing. Staining with ethidium bromide visualised the DNA marker (100–12 000 bp) and single bands of 303 bp amplicons specific for hPR (exon 4–5) from all kidney specimens and MCF7. pRShMR was not amplified by hPR-specific PCR.

addition to 35 cycles of PCR, lower cycle numbers were performed. Staining of specific PCR products was obtained from MCF7 cDNA after 25 cycles and from kidney samples after 30 cycles of PCR (data not shown). Direct sequencing (forward with the hPR sense primer and reverse with the hPR antisense primer) of the PCR product from the premenopausal kidney cortex revealed 100% sequence homology (Table 3) according to the published sequence (Misrahi *et al.* 1987). This confirmed the identity of the hPR-specific RT-PCR product in the human kidney.

Expression of different enzymes for progesterone metabolism

PCRs for steroid-metabolising enzymes were performed with different specific primer pairs (Table 1) tested with cDNA templates from human adrenals and testes as positive controls (Fig. 4). Amplicons of primer-specific calculated sizes (Table 1) were obtained for 17α-hydroxylase (527 bp), 20α-HSD (416 bp), 3α-HSD type 3 (191 bp), 3α-HSD type 2 (562 bp), 17β-HSD type 5 (335 bp), 3β-HSD type 2 (382 bp) and 5α-Red type 1 (239 bp) from both adrenals and testes. PCR products for 5α-Red type 2 (244 bp) and 5β-Red (451 bp) were synthesised only from testes but not from adrenals, and for 3β-HSD type 1 amplification could not be detected from adrenals (Fig. 4) or from testes (data not shown). PCRs with cDNA templates from human kidney cortex and medulla (premenopausal and male) and from the monkey kidney cell line CV-1 gave the following results.

17α-hydroxylase (P450c17=CYP17) The renal expression of 17α-hydroxylase (capable of hydroxylation at the angular C17 of the pregnane body) was analysed by RT-PCR slightly modified from the method described by Jose *et al.* (1999). Amplification of the expected PCR product of 527 bp was detected from cDNAs of all kidney samples as well as from CV-1 (Fig. 5A). Sequencing of the re-extracted and reamplified 527 bp PCR product

Table 3 Renal expression of PR and progesterone-metabolising enzymes. Expression analyses were performed from human kidney specimens (cortex and medulla) by RT-PCR and direct sequencing of specific amplicons from samples of one premenopausal patient. Detection is indicated by + and the homologies of the PCR product to the published sequences are given as percentage. − is used if expression (e.g. of isoenzymes) was not detected in the human kidney specimens used for analysis

	Amplification of cDNA (bp)	Found in the human kidney	Homology to published sequences (%)	References
Analysed receptor and enzymes				
hPR	303	+	100	Misrahi *et al.* (1987)
17α-hydroxylase (=CYP17)	527	+	100	Chung *et al.* (1987)
			99·8	Picado Leonard & Miller (1987)
20α-HSD (=DD1, AKR1C1)	416	+	100	Nishizawa *et al.* (2000)
3α-HSD type 3 (=DD2, AKR1C2)	191	+	100	Shiraishi *et al.* (1998)
PGFS (=3α-HSD type 2, AKR1C3)	562	+	99·8	Suzuki-Yamamoto *et al.* (1999)
17β-HSD type 5 amplicon 1	476	+	99·6	Qin *et al.* (1993)*
17β-HSD type 5 amplicon 2	335	+	99·1	Qin *et al.* (1993)*
17β-HSD type 5 amplicon 3	469	+	99·5 for complete CDS	Qin *et al.* (1993)*
17β-HSD/3α-HSD type 2			99·8 for complete CDS	Lin *et al.* (1997)
3α-HSD (=HAKRb)	—	−	—	Qin *et al.* (1993)
3β-HSD type 1	—	−	—	Dumont *et al.* (1992)
3β-HSD type 2	382	+	100	Rheaume *et al.* (1991)
5α-Red type 2		−	—	Andersson *et al.* (1991)
5α-Red type 1	168 (gene)	+	95·0 female and 99·0 male	Andersson & Russell (1990)
	168 (pseudo)	+	99·4	Jenkins *et al.* (1991)
5α-Red type 1	351 (gene)	+	99·0	Andersson & Russell (1990)
	357 (pseudo)	+	100	Jenkins *et al.* (1991)
5α-Red type 1 pseudogene	454 (pseudo)	+	100	Jenkins *et al.* (1991)
5α-Red type 1 gene	239 (gene)	+	100	Andersson & Russell (1990)
5β-Red	451 (gene)	+	89·0	Kondo *et al.* (1994)
	432 (pseudo)	+	99·0	Charbonneau & The (2001)

*Note that the sequence published for HAKRb was supposed to be 3α-HSD type 2, highly homologous to 17β-HSD type 5 (an enzyme with only two amino acid exchanges). pseudo, pseudogene.

from the premenopausal kidney cortex revealed 100% homology to the corresponding part of the CYP17 sequence (Chung *et al.* 1987) (Table 3).

For all investigated HSDs and reductases only those PCR products from premenopausal kidney of the same patient are shown in Fig. 5B and C. The same amplicons were used for sequencing.

AKR1C1 (=20α-HSD, dihydrodiol dehydrogenase 1 (DD1)) The expression of 20α-HSD (aldo-keto reductase AKR1C1) (Nishizawa *et al.* 2000) was analysed by sequencing a specific PCR product amplified with highly specific primers (Table 1) for the AKR1C1 sequence containing mismatches to sequences of other members of the AKR1C family. Amplification of the expected 416 bp product appeared in all human kidney samples as well as in CV-1 monkey kidney cells. One hundred per cent homology to the published sequence of 20α-HSD was found (Table 3).

AKR1C2 (=3α-HSD type 3, DD2, bile acid binding protein BABP) Analysing the expression of the enzyme 3α-HSD type 3 (=AKR1C2, DD2, BABP), the sequence of a PCR product amplified with primers containing unique nucleotides for 3α-HSD type 3 at their 3′-ends (Table 1), revealed 100% homology to the sequence of 3α-HSD type 3 (Shiraishi *et al.* 1998) (Table 3).

AKR1C3 (=PGFS, 3α-HSD type 2) The expression of the enzyme PGFS (=AKR1C3) with a sequence (Suzuki-Yamamoto *et al.* 1999) identical with AKR1C3, which was reported to be 3α-HSD type 2 (Khanna *et al.* 1995), was investigated by sequencing a PCR product obtained with a PGFS-specific sense primer (Table 1) and the antisense primer (17β-HSD type 5 as1★), used because of high homology to the corresponding sequence of PGFS. For the 562 bp product, 99·8% homology was found to the PGFS sequence (Table 3) with G instead of A (312th nucleotide (nt) of the CDS), which does not alter the amino acid sequence.

17β-HSD type 5 similar to 3α-HSD (clone of human aldo-keto reductase b (HAKRb)) As the human enzyme 17β-HSD type 5 possesses a high 20α-HSD activity that inactivates progesterone to 20α-DH-P (Dufort *et al.* 1999), we analysed the expression of 17α-HSD type 5 in

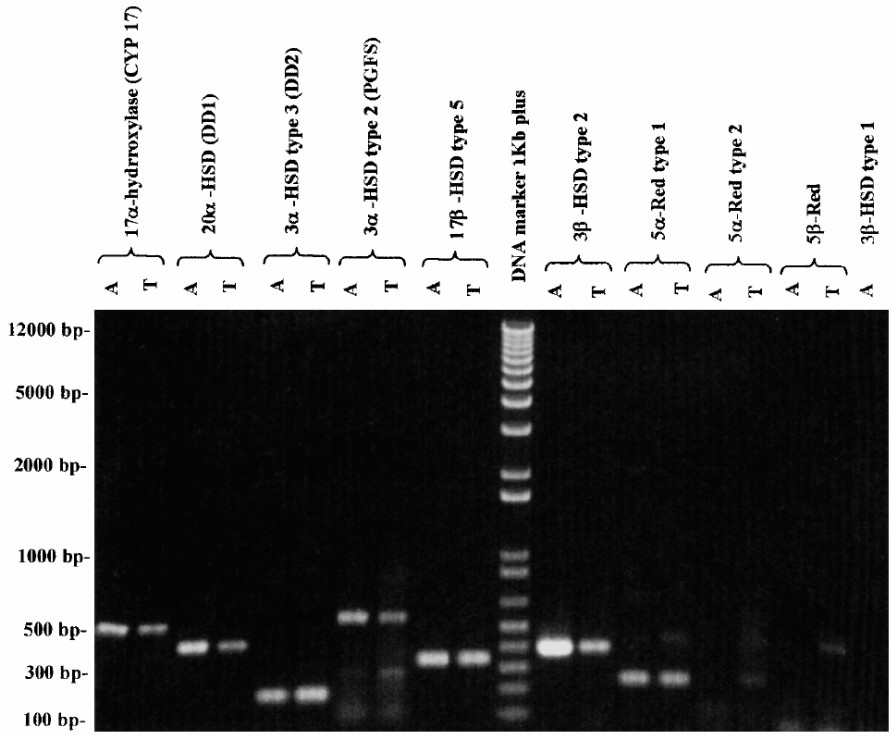

Figure 4 High-stringency PCR controls for steroid-metabolising enzymes. cDNAs from adrenals (A) and testes (T) as positive controls were amplified with specific primer pairs for different steroid-metabolising enzymes. PCR products of expected size of 527 bp for 17α-hydroxylase, 416 bp for 20α-HSD, 191 bp for 3α-HSD type 3, 562 bp for 3α-HSD type 2, 335 bp for 17β-HSD type 5, 382 bp for 3β-HSD type 2, and 239 bp for 5α-Red type 1 were obtained from both adrenals and testes. Amplifications of 244 bp for 5α-Red type 2 and 451 bp for 5β-Red were achieved only from testes, and a specific amplicon for 3β-HSD type 1 could not be shown.

the human kidney. Amplifications of three overlapping parts originating from exon 1–5, 4–7 and 6–9 covering nearly the complete CDS (Table 1) were performed with primers according to the homologous sequence of 3α-HSD clone HAKRb (Qin *et al.* 1993) encoding an enzyme differing only in the 75th and 175th amino acid from 17β-HSD type 5. All three expected PCR products of 476, 335 and 469 bp (Table 3) were amplified from cDNAs of all human kidney samples and CV-1 cells as well, the latter missing only the PCR product of the last segment. This suggests differences in human and monkey sequences located 3′ to the stop codon TAA for translation. Three 17β-HSD type 5-specific PCR products were used for complete sequencing. We found differences with respect to the sequence of HAKRb (Qin *et al.* 1993): G instead of T (222nd nt), G instead of A (495th nt), G instead of C (702nd nt), all of which do not alter the amino acid sequence. However, two additionally identified nucleotide substitutions resulted in amino acid changes; A instead of G (223rd nt) changing glutamic acid (75th amino acid) to lysine, and G instead of C (525th nt) changing isoleucine (175th amino acid) to methionine (such as in all other members of the AKR1C family). Our sequencing data show 99·5% homology to the CDS of HAKRb (Qin *et al.* 1993); 99·8% homology was found to the sequence of a recombinant 3α-HSD type 2 with 17β-HSD activity (Lin *et al.* 1997), identified as 17β-HSD type 5 (Penning 1999). Our sequence differed only in the 312th nt with G (such as in the sequence of HAKRb) instead of A, and in the 855th nt with G (such as in the sequences of PGFS and HAKRb) instead of A, both conserving the amino acid sequence of 17β-HSD type 5.

HAKRb (=3α-HSD) The expression of an enzyme structurally related to 3α-HSD with a sequence according to clone HAKRb (Qin *et al.* 1993) with nearly the same genomic sequence found for 3α-HSD type 2 (Khanna *et al.* 1995) was investigated by using a highly specific antisense primer (Table 1) ending 3′ with two unique nucleotides for HAKRb. The expected 248 bp PCR product was not amplified in any of our kidney samples even at low-stringency annealing temperature for this

specific primer. Therefore, an expression of an enzyme encoded by the sequence described by Qin *et al.* (1993) for 3α-HSD clone HAKRb could be excluded.

3β-HSD type 1 and 3β-HSD type 2 For 3β-reduction of the keto group in position 3 of steroids, the expressions of two different isoforms of the enzyme 3β-HSD were analysed with 3β-HSD type 1- and 3β-HSD type 2-specific primer pairs (Table 1). Only PCR products with the expected size of 382 bp for 3β-HSD type 2-specific amplification were obtained (Table 3). Sequencing of the re-extracted and reamplified PCR product (Fig. 5C) revealed 100% homology to the sequence of 3β-HSD/delta-4-delta-5 isomerase (Rheaume *et al.* 1991). Therefore, we conclude that only the isoform 3β-HSD type 2 is expressed in the human kidney, while the expression of 3β-HSD type 1 could not be detected in any of our kidney samples (Table 3).

5α-Red type 2, 5α-Red type 1 gene and pseudogene For ring A reduction of steroids, producing 5α-DH metabolites, the expression of the reductases 5α-Red type 1 and 5α-Red type 2 was examined. 5α-Red type 2-specific primers (Table 1) did not amplify the expected 244 bp product from any renal sample (Table 3), but PCR with the specific primer pair for 5α-Red type 1 (located in exon 1 and 2) resulted in amplification of the expected 168 bp PCR product with 99·4% homology (Table 3) to the published sequence of the 5α-Red-pseudogene (located on chromosome X) (Jenkins *et al.* 1991). There was only one nucleotide substitution: G (gene-specific at this position) instead of A. According to the cDNA sequence (Andersson & Russell 1990) from the gene of 5α-Red type 1 (located on chromosome 5), our main

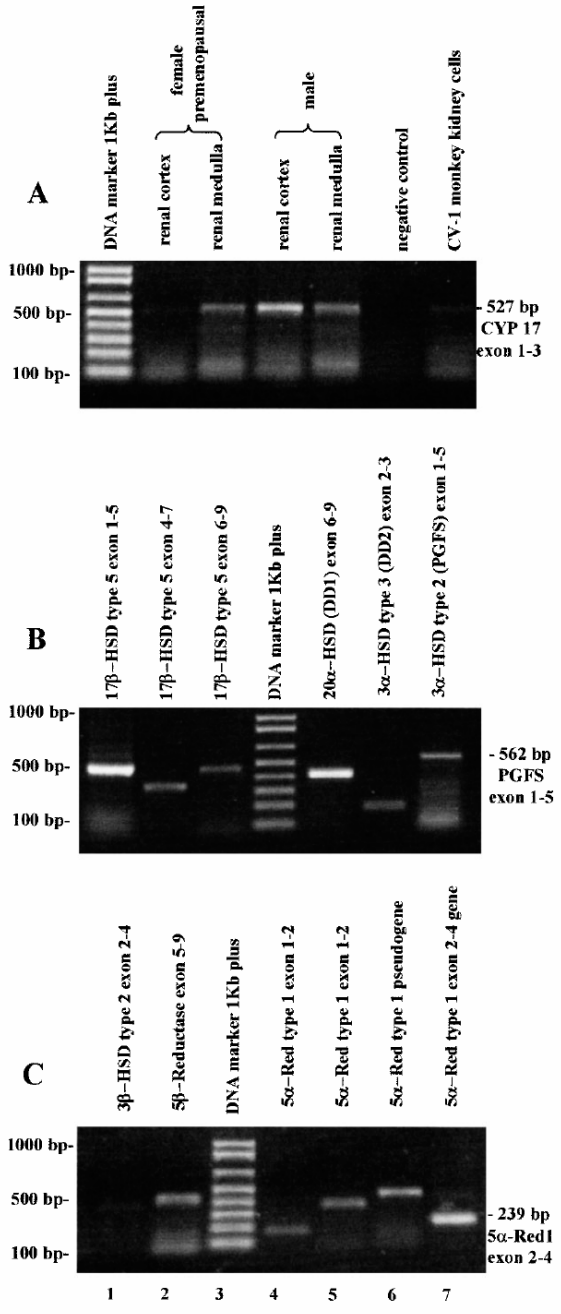

Figure 5 RT-PCR analysis for the expression of progesterone-metabolising enzymes. (A) 17α-hydroxylase (CYP17). cDNAs from premenopausal and male renal cortex and medulla and from CV-1 monkey kidney cells (control) were amplified in the first step with specific primers 17α-hydroxylase sense and antisense. The negative control without cDNA did not give any staining while all kidney samples stained positively for the expected 527 bp amplicon with a smear of smaller fragments. Therefore the DNA corresponding to the band of the correct size was re-extracted from the gel and reamplified for sequencing. (B) HSDs capable of 20α-reduction. Three sets of primers were used for overlapping amplicons of 17β-HSD type 5 covering nearly the complete CDS and each one single primer pair for 20α-HSD, 3α-HSD type 3, 3α-HSD type 2. Specific PCRs were performed with cDNAs from female and male kidney cortex and medulla and CV-1 as well. Here premenopausal samples used for sequencing are shown. Three specific PCR products for 17α-HDS type 5: amplicon 1 (exon 1–5) 476 bp, amplicon 2 (exon 4–7) 335 bp, and amplicon 3 (exon 6–9) 469 bp, each one single specific PCR products for 20α-HSD (exon 6–9) 416 bp, 3α-HSD type 3 (exon 2–3) 191 bp and 3α-HSD type 2 (exon 1–5) 562 bp. (C) 3β-HSD type 2 for 3β-reduction and reductases for 5β- and 5α-reduction. Specific amplicons of cDNA samples from premenopausal kidney specimens used for sequencing are shown for 3β-HSD type 2 (exon 2–4) 382 bp (lane 1), 5β-Red (exon 5–9) 451 bp (lane 2), DNA marker (100–1000 bp shown) (lane 3), 5α-Red type 1 (exon 1–2) 168 bp (lane 4), 5α-Red type 1 (exon 1–2 amplified with a sense primer located preATG) 351 bp and/or 357 bp for the gene and/or pseudogene transcript (lane 5), 5α-Red type 1 pseudogene-specific 454 bp (lane 6), and 5α-Red type 1 gene-specific (exon 2–4) 239 bp (lane 7).

sequence revealed only 95·0% homology (Table 3). In the chromatogram obtained by direct sequencing of the amplicon, additional underlying peaks of gene-specific nucleotides with lower concentration were also detectable at the positions of pseudogene-specific nucleotide substitutions. Therefore, in the sample of the premenopausal kidney cortex, mainly transcription of the pseudogene from chromosome X besides lower expression of the gene for 5α-Red type 1 could be found. Because of this phenomenon, the PCR product from a male kidney medulla cDNA was sequenced additionally. In the direct sequencing data, 99·0% homology to the sequence of 5α-Red type 1 gene (Table 3) was found with an underlying sequence of the pseudogene at lower levels. For analysing the region with six duplicated nucleotides in the pseudogene directly following the start codon ATG, the sense primer (5α-Red type 1 preATG sense) located 5′ of the CDS matching both sequences and the antisense primer (5α-Red type 1 antisense) were used for amplification. Sequencing of the PCR product from the premenopausal renal medulla (Fig. 5C, lane 5) again revealed high homologies (Table 3) to both the expected 351 bp for gene-specific and 357 bp for pseudogene-specific sequences (including the six additional nucleotides). Combination of the common sense primer (5α-Red type 1 preATG sense) with a pseudogene-specific antisense primer (5α-Red type 1 pseudo antisense) and further amplification of the PCR product in combining the same pseudogene-specific antisense primer with a pseudogene-specific sense primer (5α-Red type 1 pseudo sense) by a second (semi-nested) PCR resulted in the expected 454 bp product (Fig. 5C, lane 6). Sequencing of the amplified cDNA from the premenopausal renal medulla revealed a uniform sequence with 100% homology for the pseudogene (Table 3) including the internal stop codon TGA unique to the pseudogene. Therefore, transcription of the pseudogene in the human kidney can be assumed, but translation would not result in a full-length protein because of premature termination. For analysing the 5α-Red type 1 gene expression, a gene-specific primer pair ending 3′ with nucleotides unique only to the gene (5α-Red type 1 gene sense and antisense) (Table 1) was used for amplification of a uniform PCR product. The amplified expected 239 bp product (Fig. 5C, lane 7) was 100% homologous to the gene-specific sequence of 5α-Red type 1 (Andersson & Russell 1990) (Table 3). Therefore, the expression of the isoform 5α-Red type 1 was found in the human kidney, besides transcription of the pseudogene, while 5α-Red type 2 expression could not be detected.

5β-Red (=AKR1D1) gene and pseudogene For the synthesis of 5β-reduced metabolites, expression analyses were performed for the enzyme 5β-Red (AKR1D1). PCR products of about 430–450 bp using the primer pair 5β-Red sense and antisense (Table 1) were obtained (Fig. 5C). According to the sequence for the 5β-Red cDNA (Kondo *et al.* 1994), an amplicon of 451 bp was expected. By direct sequencing we found 99·0% homology (Table 3) to the sequence of the 5β-Red pseudogene (Charbonneau & The 2001) (reverse complement sequence located on chromosome 1), missing 19 nucleotides in the amplified cDNA of 432 bp compared with 451 bp from the 5β-Red gene transcript. To the 5β-Red gene cDNA sequence (Kondo *et al.* 1994) only 89·0% homology was found (Table 3). Another direct sequencing from a sample of another female patient revealed highly homologous sequences specific for the pseudogene and the gene at the same time. Therefore, the transcription of the pseudogene and the expression of the enzyme 5β-Red was confirmed in the human kidney.

Discussion

This study examined mechanisms by which the MR may be protected against binding and antagonism of progesterone, which binds to the MR with the same or even higher affinity, but confers only little transcriptional activity (Rupprecht *et al.* 1993*a*, Myles & Funder 1996). Therefore progesterone is assumed to be an MR antagonist. We found that progesterone has a higher antagonistic than agonistic function at the MR (Quinkler *et al.* 2002). It is still unclear how the mineralocorticoid function of aldosterone can be maintained especially at higher progesterone than aldosterone levels, e.g. during the luteal phase and pregnancy, when progesterone exceeds aldosterone concentrations 100-fold in the third trimester (Rosenthal *et al.* 1969).

One protective mechanism could be achieved by an additional binding site for progesterone in renal cells, e.g. the PR for high-specific progesterone binding , resulting in a sequestration of progesterone. This could enable binding of aldosterone to the MR. Therefore, we investigated the renal expression of the hPR. We found the expression of the hPR in kidney specimens from female (pre- and postmenopausal) and male patients in cortical as well as in medullary tissue preparations. The function of the receptor protein, tested in binding assays with the specific ligand R5020 and Scatchard plot analysis, revealed K_d values of about 0·8 nM (mean of cortex and medulla) in kidney specimens compared with 0·98 nM in the target tissue myometrium, consistent with the published K_d of 0·89 nM for the myometrial hPR (Keightley 1979). These K_d values for binding of R5020 characterise the same kind of receptor (hPR) in the myometrium and in the human kidney, supporting our hypothesis of renal hPR expression. The identity of the hPR was further tested by immunodetection. In tissue sections, a nuclear localisation of hPR was detected with the IgG monoclonal PgR 636 predominantly in epithelial cells of distal tubules, the mineralocorticoid target cells of the kidney. Western blots

(differentiating PR-A and PR-B) were performed for isoform analysis of hPR by immunodetection with the hPR-specific IgG1 monoclonal antibody NCL-PGR-312. A protein of about 117 kDa corresponding to hPR-B (Sheridan *et al.* 1989, Kumar *et al.* 1998) was found in protein extracts from male and female kidney specimens, but a specific immunoreaction with a protein of the size corresponding to the isoform hPR-A (Kastner *et al.* 1990*b*) could not be shown. This is of special interest because hPR-A is discussed as a co-repressor of hMR expression and function (McDonnell *et al.* 1994). Therefore a renal downregulation of hMR function by PR-A is not likely. In addition, our results of RT-PCRs from RNA of kidney cortex and medulla specimens from male and female patients and sequencing data with 100% homology to hPR cDNA sequence (Misrahi *et al.* 1987), confirmed the identity of hPR and the renal transcription of the hPR gene. Therefore, the expression of hPR could be shown for the first time in the human kidney.

Renal progesterone-specific functions via hPR remain to be investigated. To date it is not known whether there is an additional influence on the mineralocorticoid function by a possibility of heterodimerisation, e.g. of hPR-B with the hMR co-localised in epithelial cells of distal tubules in the human kidney. Rather low levels of hPR in the kidney of postmenopausal origin were detected by Scatchard plot analyses, reflecting the mean of all cells in renal cortex or medulla. As only in a small number of cells was PR detected by immunohistology, especially in epithelia of distal tubules, local PR levels may be able to compete with MR for progesterone binding. Whether the expression of hPR would be raised in kidneys of premenopausal women by oestrogen for compensation of elevated progesterone could not be analysed because tissues from females during different cycle stages and/or pregnancy were not available. To discuss the role of the renal hPR for the hMR specificity, experiments investigating the number of both receptors and the co-localisation in renal cells would be helpful.

Additional mechanisms for protecting the hMR in the kidney against binding of progesterone appear to be essential. Another way of reducing progesterone binding to MR is the metabolism of progesterone to derivatives with lower affinity to the MR. More than 50% of progesterone is converted to 17α-OH-P, 20α-DH-P, 17α-OH,20α-DH-P, 5α-DH-P, 5β-DH-P, 3β,5α-TH-P, 3α,5α-TH-P, 3β,5β-TH-P, 20α-DH,5α-DH-P and 20α-DH,3β,5α-TH-P in the human kidney (Quinkler *et al.* 1999, 2001). These metabolites exhibit reduced affinity to the MR depending on hydroxylated and/or reduced groups (Quinkler *et al.* 2002). For different pathways of progesterone metabolism (Fig. 6), we examined the expression of corresponding steroid-converting enzymes in the human kidney by RT-PCR.

Metabolism of progesterone to 17α-OH-P is achieved by 17α-hydroxylase (CYP17). The expression of this

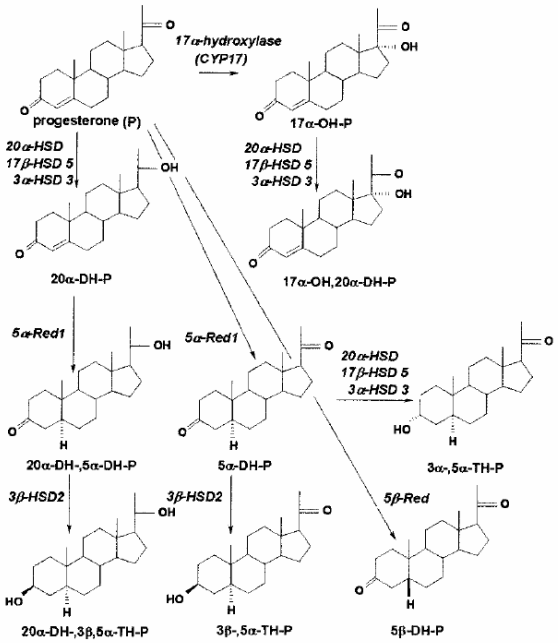

Figure 6 Scheme for progesterone metabolism with enzymes identified in the human kidney. Steroid-metabolising enzymes, confirmed by sequencing of RT-PCR products from human kidney specimens, are shown in different pathways of progesterone.

enzyme was found in renal cortex and medulla. One of 527 analysed nucleotides differed from the published sequence of exon 1 (Picado-Leonard & Miller 1987) of the gene of CYP17, but this difference had been previously described by Chung *et al.* (1987). Therefore, in the kidney, hydroxylation by CYP17 of progesterone to 17α-OH-P with lower affinity to the hMR is likely. For the reduction of progesterone to 20α-DH-P we found the expression of different enzymes in our kidney specimens. Direct sequencing of a 20α-HSD-specific RT-PCR product from renal RNA preparations revealed 100% homology to the sequence for 20α-HSD (AKR1C1) (Nishizawa *et al.* 2000). By sequencing of three overlapping 17β-HSD type 5-specific amplicons covering nearly the complete CDS, we identified RNAs specific for the enzyme 17β-HSD type 5 with a strong 20α-Red activity (Dufort *et al.* 1999). Amplification with a PGFS-specific sense primer resulted in a PCR product with 99·8% homology to the published sequence (Suzuki-Yamamoto *et al.* 1999) for PGFS (AKR1C3). This amplicon contained identical sequences as obtained by the first 17β-HSD type 5-specific PCR. Comparison of the complete CDS of PGFS (AKR1C3) and the sequence of 3α-HSD type 2 (AKR1C3) transcript from chromosome 10p15–p14 (deduced from six published sequences in GeneBank accession No. 003739·2) exhibits about

99·7% homology. Our complete sequencing data of the CDS for 17β-HSD type 5 reveal also 99·7% homology to both AKR1C3 sequences described for PGFS and 3α-HSD type 2. Therefore we think that the sequence of the renal cDNA we found encodes 17β-HSD type 5. It is probably the same enzyme as AKR1C3, as recently described (Penning *et al.* 2001). 3α-HSD type 3 (AKR1C2, DD2, BABP)-specific RNA with 100% homology (Shiraishi *et al.* 1998) was also identified in our kidney specimens, but this enzyme shows only weak activity of 20α-reduction. Therefore the enzymes 20α-HSD and 17β-HSD type 5 are the main candidates for the 20α-reduction of progesterone and 17α-OH-P, while 3α-HSD type 3 may contribute to the 3α-reduction of 5α-DH-P to 3α,5α-TH-P in the human kidney. 3β-Reduced progesterone metabolites are synthesised in the kidney by 3β-HSD type 2. The expression was found in renal cortex and medulla by RT-PCR with 100% homology to the published sequence (Rheaume *et al.* 1991). As 3β-HSD type 1 was not detected, the isoform 3β-HSD type 2 must be responsible for renal formation of 3β,5α-TH-P. For synthesis of this metabolite and direct 5α-reduction of progesterone, the expression of the isoform 5α-Red type 1 was found in the kidney. The amplicon of gene-specific RT-PCR exhibits 100% sequence homology to the 5α-Red type 1 gene-specific CDS located on chromosome 5 (Andersson & Russell 1990). Additionally a pseudogene-specific RT-PCR product reveals 100% homology to the pseudogene with X-chromosomal location (Jenkins *et al.* 1991) as well. The role of the transcription of the intronless pseudogene (Mighell *et al.* 2000) with a stop codon in the region corresponding to the CDS, in the context of simultaneous gene expression, remains to be investigated. For the renal 5β-reduction of progesterone, we found the expression of the enzyme 5β-Red (AKR1D1) from the gene located on chromosome 7 q32–q33 and also the transcription of the pseudogene located on chromosome 1 q23–q25 (reverse complement sequence) (Charbonneau & The 2001) in the human kidney. There may be a regulatory role by transcription of the reverse complement sequence (antisense RNA) of 5β-Red gene on expression of the enzyme 5β-Red.

In conclusion, we produced evidence for two mechanisms for the mineralocorticoid function of aldosterone at the renal hMR in the presence of elevated progesterone. Binding of progesterone to the renal MR may be partly prevented by competitive binding to the PR, and the concentration of free progesterone should be lowered by steroid-metabolising enzymes producing progesterone metabolites with reduced affinity to the MR in renal cells (Quinkler *et al.* 2002).

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3.3 Androgensynthese in der Niere

11. **Quinkler M**, Bumke-Vogt C, Meyer B, Bähr V, Oelkers W, Diederich S.
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The Human Kidney Is a Progesterone-Metabolizing and Androgen-Producing Organ

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Progesterone (P) is a potent antagonist of the human mineralocorticoid receptor (MR) *in vitro*. We have previously demonstrated effective downstream metabolism of P in the kidney. This mechanism potentially protects the MR from P action. Here, we have investigated the expression and functional activity of steroidogenic enzymes in human kidney. RT-PCR analysis demonstrated the expression of 5α-reductase type 1, 5β-reductase, aldo-keto-reductase (AKR) 1C1, AKR1C2, AKR1C3, 3β-hydroxysteroid dehydrogenase (3β-HSD) type 2, and 17α-hydroxylase/17,20-lyase (P450c17). The presence of 3β-HSD type 2 and P450c17 indicated that conversion of pregnenolone to dehydroepiandrosterone (DHEA) and to androstenedione may take place effectively in kidney. To investigate this further, we incubated kidney subcellular fractions with radiolabeled pregnenolone. This resulted in efficient formation of DHEA from pregnenolone, indicating both 17α-hydroxylase and 17,20-lyase activities exerted by P450c17. Radiolabeled DHEA was converted via androstenedione, androstenediol, and testosterone, indicating both 3β-HSD type 2 activity and 17β-HSD activity. In addition, the conversion of testosterone to 5α-dihydrotestosterone was detectable, indicating 5α-reductase activity. In conclusion, we verified the expression and functional activity of several enzymes involved in downstream metabolism of P and androgen synthesis in human kidney. These findings may be critical to the understanding of water balance during the menstrual cycle and pregnancy and of sex differences in hypertension. (*J Clin Endocrinol Metab* 88: 2803–2809, 2003)

PROGESTERONE (P) IS a strong mineralocorticoid receptor (MR) antagonist *in vitro* due to its high binding affinity to the MR (1–4). However, there does not appear to be a significant anti-MR effect *in vivo* even with high P concentrations, *e.g.* luteal phase (30–110 nmol/liter) and pregnancy (320–700 nmol/liter) (5, 6). Until recently, it was unknown how aldosterone maintained its function as an effective MR agonist in the presence of a 100-fold excess of P and why there were not more anti-MR effects, *e.g.* enhanced diuresis, lowering of blood pressure, and electrolyte disturbances. Previous explanations were not satisfactory, *e.g.* higher plasma protein binding of P or higher instability of the P-MR complex. We have recently shown that the kidney is able to effectively metabolize P to downstream metabolites, such as 17α-hydroxyprogesterone (17α-OH-P), 20α-dihydroprogesterone (20α-DH-P), and ring A reduced metabolites (7, 8), which possess weaker inhibitory activity than P at the MR (4). This may be the mechanism responsible for protection of the MR from P action, similar to the inactivating metabolism of cortisol to cortisone by 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 (9–11).

This study investigated which enzymes are responsible for downstream conversion of P in the human kidney. Furthermore, the enzymes identified suggested that these enzymes may also be involved in the conversion of other steroid substrates, *e.g.* precursors of androgen synthesis, in human kidney. Therefore, we explored the functional activity of steroidogenic enzymes expressed in human kidney and potentially involved in androgen synthesis.

Abbreviations: AKR, Aldo-keto-reductase; 20α-DH-P, 20α-dihydroprogesterone; DHEA, dehydroepiandrosterone; DH-T, dihydrotestosterone; 3α-diol, 3α-androstanediol; 4-dione, androstenedione; 3β-HSD, 3β-hydroxysteroid dehydrogenase; MR, mineralocorticoid receptor; 17α-OH-P, 17α-hydroxyprogesterone; 17α-OH-Preg, 17α-hydroxypregnenolone; P, progesterone; P450c17, 17α-hydroxylase/17,20-lyase; Preg, pregnenolone; T, testosterone; 3β,5α-TH-P, 5α-pregnan-3β-ol-20-one; TLC, thin layer chromatography; V_{max}, maximal reaction velocity.

Materials and Methods

Radiolabeled and unlabeled steroids

[7-^{3}H]Pregnenolone ([7-^{3}H]Preg; 10–25 Ci/mmol), [4-^{14}C]P (0.02 mCi/ml), and [4-^{14}C]dehydroepiandrosterone ([4-^{14}C]DHEA; 0.02 mCi/ml) were purchased from NEN Life Science Products (Boston, MA).

Unlabeled P (4-pregnen-3,20-dione), deoxycorticosterone, 20α-DH-P (4-pregnen-20α-ol-3-one), 5α-DH-P (5α-pregnan-3,20-dione), 3β,5α-TH-P (5α-pregnan-3β-ol-20-one), 20α-DH,5α-DH-P (5α-pregnan-20α-ol-3-one), 16α-OH-P (4-pregnen-16α-ol-3,20-dione), 5β-DH-P (5β-pregnan-3,20-dione), 20α-DH,3β,5α-TH-P (5α-pregnan-3β,20α-diol), 20α-DH, 3α,5α-TH-P (5α-pregnan-3α,20α-diol), 3α,5α-TH-P (5α-pregnan-3α-ol-20-one), 11α-OH-P (4-pregnen-11α-ol-3,20-dione), 11β-OH-P (4-pregnen-11β-ol-3,20-dione), 20α-DH,3α,5β-TH-P (5β-pregnan-3α,20α-diol), androstenedione (4-dione; 4-androsten-3,17-dione), DHEA (5-androsten-3β-ol-17-one), Preg (5-pregnen-3β-ol-20-one), 17α-hydroxypregnenolone (17α-OH-Preg; 3β,17α-dihydroxy-5-pregnen-20-one), testosterone (T; 4-androsten-17β-ol-3-one), estrone (1,3,5(10)-estratrien-3-ol-17-one), 17β-estradiol (1,3,5(10)-estratrien-3α,17β-diol), 5α-dihydrotestosterone (5α-DH-T; 5α-androstane-17β-diol-3-one), 5β-DH-T (5β-androstane-17β-diol-3-one), 3α-androstanediol (3α-diol; 5α-androstane-3α,17β-diol), androstanedione (5α-androstane-3,17-dione), androstenediol (5-androstene-3β,17β-diol), and androsterone (5α-androstane-3α-ol,17β-one) were obtained from Sigma-Aldrich Corp. (St. Louis, MO). 17α-OH-P (4-pregnen-17α-ol-3-one) was purchased from Makor Chemicals Ltd. (Jerusalem, Israel). 17α-OH,20α-DH-P (4-pregnen-17,20α-diol-3-one) and 3α,5β-TH-P (5β-pregnan-3α-ol-20-one) were obtained from Paesel & Lorei (Hanau, Germany), and 6β-OH-P (4-pregnen-6β-ol-3,20-diol) was obtained from Steraloids, Inc. (Wilton, NH).

Preparation of subcellular fractions from human kidney tissue

Human kidney specimens from tumor nephrectomies of postmenopausal women were obtained and prepared as described previously (7,

8). Ethical approval was granted by the local ethic committee. Renal cortex and medulla were divided macroscopically, cut into small pieces, and homogenized for preparation of subcellular fractions or for RNA preparation. The preparation of subcellular fractions was performed as described by Lakhsmi *et al.* (12). Total protein content was estimated by the Bradford method (Bio-Rad Laboratories, Inc., Munich, Germany).

Incubations with kidney subcellular fractions

We performed preliminary studies for time kinetics, protein kinetics, and cosubstrate preferences (data not shown) to determine optimum conditions for incubation of subcellular fractions. Both cytosolic and microsomal incubations were carried out in 1 ml 0.01 M sodium phosphate buffer (pH 7.0 for reduction and pH 8.1 for oxidation) and, in addition, a coenzyme-regenerating system (10^{-2} M glucose-6-phosphate, 10 U glucose-6-phosphate-dehydrogenase; Roche, Mannheim, Germany). All incubations were carried out at 37 C in a shaking water bath; all experiments were performed in triplicate.

Incubation assay for the cytosolic fraction. We incubated 4 mg cytosolic protein from kidney cortex and medulla with 10^{-3} mol/liter NADPH and 200,000 cpm [4-^{14}C]P and [4-^{14}C]DHEA, respectively, for 120 min. We investigated the following unlabeled steroids as substrates (10^{-4} M) under the same conditions using NADPH as a cosubstrate for reduction, $NADP^+$ for oxidation of 3α,5β-TH-P, 20α-DH,3α,5β-TH-P, 20α-DH,5β-DH-P, 20α-DH-P, T, 5α-DH-T, 3α-diol, 4-dione, and estrone. For oxidation of 3α-diol, NAD^+, rather than $NADP^+$, was added. Enzyme kinetic analysis for 20α-reduction of P was performed using increasing concentrations of unlabeled P (10^{-8}–10^{-5} M) in addition to radiolabeled P within the linear time range of the enzymatic reaction (40 min of incubation).

Incubation assay for the microsomal fraction. One milligram of microsomal protein isolated from kidney cortex and medulla was incubated with 10^{-3} M NADPH (for reduction, pH 7.0) or NAD^+/$NADP^+$ (for oxidation, pH 8.1); 200,000 cpm [4-^{14}C]P, [7-^{3}H]Preg, or [4-^{14}C]DHEA were added, and incubations were carried out for 60, 120, and 120 min, respectively, thus ensuring that incubations were within the linear time frame of the enzymatic reactions. In addition, 10^{-4} M 4-dione, T, Preg, and DHEA were incubated with the microsomal fraction under the same conditions for 120 min with NADPH as cosubstrate

Steroid extraction and detection of steroids by thin layer chromatography (TLC)

The incubations were stopped, and steroids were extracted from the incubations with methylacetate and separated by two-dimensional TLC (first dimension, 50 min in 35/65 methylacetate/ethylendichloride; second dimension with 25:75 hexanol/hexane for 210 min) as described previously (7, 8) and shown in Fig. 1. For detection of T, 5α-DH-T, and 5β-DH-T from microsomal incubations, we used one-dimensional TLC with 25:75 hexanol/hexane for 210 min.

RNA preparation and RT-PCR

Total RNA was prepared from four human postmenopausal kidneys using the RNeasy MIDI kit (Amersham Pharmacia Biotech, Freiburg, Germany). Single-stranded cDNA was synthesized by Superscript II-RT (Life Technologies, Inc., Karlsruhe, Germany) and random hexamer primers, using 2.5 μg total RNA as a template. cDNA (40 ng) was then subjected to PCR amplification with 12 pmol/liter specific primers for the steroidogenic enzymes of interest (TIB MOLBIOL, Berlin, Germany; GENSET SA, Paris, France) overspanning at least one intron (Table 1) in a volume of 25 μl Tris-buffer with 1 U *Taq* polymerase. PCR conditions were 35 cycles for 45 sec at 94 C, for 1 min at 55–65 C (dependent on the melting temperature of the respective primer pairs), and for 1.5 min at 72 C. Each RT-PCR analysis was performed using an additional negative control and two positive controls, human adrenal cortex and human testis (each 40 ng cDNA; Fig. 2). RT-PCR products were gel-purified and subjected to direct sequencing for verification.

For specific identification of 17β-HSD type 5 [aldo-keto-reductase (AKR) 1C3], we used three forward and reverse primers spanning across all nine exons of the gene (Table 1). The sequence of 17β-HSD type 5 (AKR1C3) differs only in amino acids 75 and 175 from the sequence of 3α-HSD type 2 (13, 14). These two different amino acids at positions 75 and 175 do not change the enzymatic properties, and it is suggested that these are identical enzymes (13, 15, 16).

Statistics

For the calculation of the Michaelis-Menten constant (K_m) and the maximal reaction velocity (V_{max}), we used the Eadie-Hofstee transformation. In addition, the intrinsic clearance value ($CL_{int} = V_{max}/K_m$) was calculated.

Results

Conversion of P and its metabolites

To characterize the enzymes involved in downstream conversion of P, we incubated subcellular fractions of human kidney with labeled P or unlabeled P metabolites. In the cytosolic fraction we found more than 80% conversion of P to 20α-DH-P, the only metabolite found in cytosol, after 4 h of incubation. The K_m for this reaction was 11.2 μmol/liter, the V_{max} was 19.4 nmol/min·mg, and the intrinsic clearance value (V_{max}/K_m) was 1.63 ml/min·mg. Consistent with the observed efficient conversion of P to 20α-DH-P, we detected

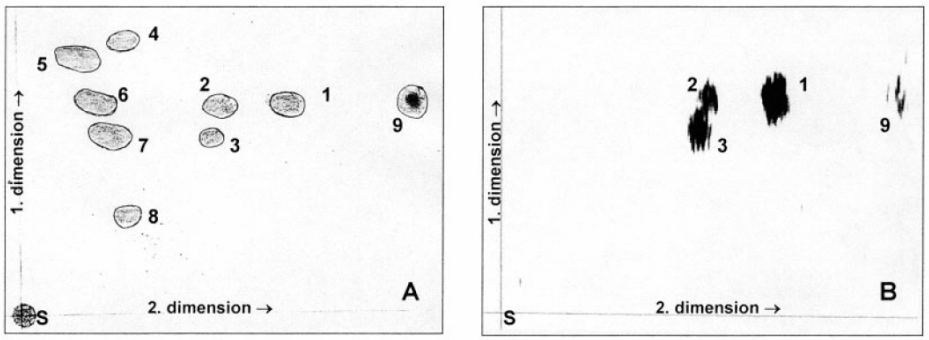

FIG. 1. Separation of steroids with two-dimensional TLC. The first dimension was run with 35:65 methylacetate/ethylendichloride for 100 min; the second dimension run was performed with 25:75 hexanol/hexane for 210 min. This figure shows the incubation of microsomes from postmenopausal renal cortex with [^{3}H]Preg. A, TLC plate after staining with Liebermann-Burchard reagent and heating, revealing the unlabeled control steroids (no. 1–7). B, ^{3}H scan of the same TLC plate. 1) Preg; 2) DHEA; 3) 17α-OH-Preg; 4) P; 5) 4-dione; 6) 17α-OH-P; 7) T; 8 and 9) unknown substances. S, Starting point.

TABLE 1. Gene-specific forward and reverse primers, the location of the primers on their genes, and the resulting amplified DNAs

Genes (enzymes)	Sequence of primers used (forward primer) (reverse primer)	Location of primers	Length of product (bp)
P450c17	5′-TCTCTTGCTGCTTACCCTAG 5′-TCAAGGAGATGACATTGGTT	Exon 1–3	527
HSD3B1 (3β-HSD type 1)	5′-GATCATCCGCCTCCTGGTG 5′-GGGTGCCGCCGTTTTTCA	Exon 2–4	485
HSD3B2 (3β-HSD type 2)	5′-GATCGTCCGCCTGTTGGTG 5′-CTCTTCTTCGTGGCCGTTCTGGATGAT	Exon 2–4	382
SRD5A1 (5α-reductase type 1)	5′-CAGATCCCCGTTTTCTAATAGG 5′-AAACGTGAAGAAAGCAAAAGC	Exon 2–4	239
SRD5A2 (5α-reductase type 2)	5′-GCCCGGCAGCCCCTCTCC 5′-CACCCAAGCTAAACCGTATGTCTG	Exon 1–2	244
AKR1D1 (5β-reductase)	5′-AACAAGCCAGGACTCAAACACAAG 5′-CTCCCGGCAGTCAGTATTCATCA	Exon 5–9	451
AKR1C1 (20α-HSD)	5′-GGGATCCCACCGAGAAGAACC 5′-TAACCAGAGGCGATGTGTCCAGTC	Exon 6–9	416
AKR1C2 (3α-HSD type 3)	5′-TAAAAGTAAAGCTCTAGAGGCCGTC 5′-ACTCTGGTCGATGGGAATTGCT	Exon 2–3	191
AKR1C3 (17β-HSD type 5)	5′-ACTTCATGCCTGTATTGGGATTTG 5′-CTGCCTGCGGTTGAAGTTTGATA	Exon 1–5	476
	5′-GGAGGCCATGGAGGAGTGTAAGGA 5′-GGTAGCGCAGGGCAATCAGG	Exon 4–7	335
	5′-AACGAGACAAACGATGGGTGGAC 5′-GTATTTCTGGCCTATGGAGTGAGC	Exon 6–9	469
(PGFS)	5′-ATTCCGGGCAGCAGCAAACA 5′-CTGCCTGCGGTTGAAGTTTGATA	Exon 1–5	562
17β-HSD type 3	5′-TCCTGAACGCACCGGATGAAAT 5′-TACCTGACCTTGGTGTTGAGCTTCAG	Exon 5–11	505
Cytochrome b5	5′-GCTCGACGGGGCTGTGT 5′-CTGCGCTGACTTCTGAGGAGGTGT	Exon 1–5	480

P450c17, 17α-Hydroxylase/17,20-lyase; AKR, aldo-keto-reductase; HSD, hydroxysteroid dehydrogenase; AKR1C1, 20α-HSD-DD1, dihydrodiol dehydrogenase type 1; AKR1C2, 3α-HSD type 3 = DD2 = BABP (bile acid binding protein); AKR1C3, 17β-HSD type 5 = 3α-HSD type 2 = PGFS (prostaglandin F synthase).

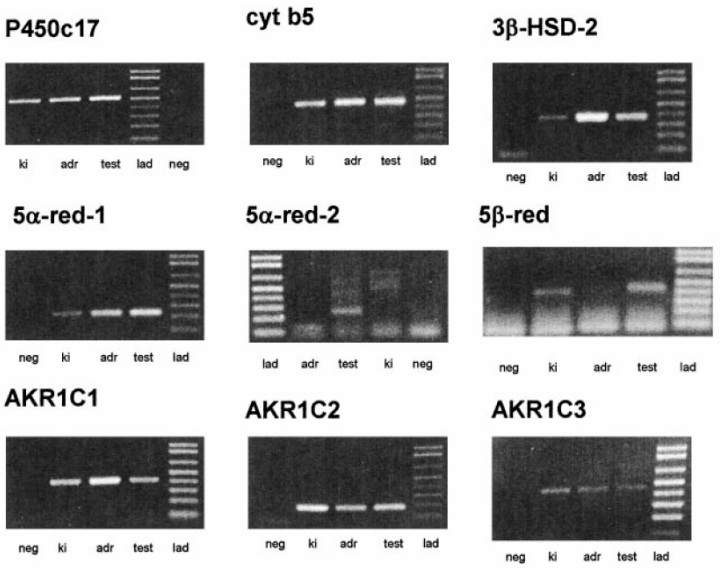

FIG. 2. RT-PCR analysis of the expression of different enzymes in postmenopausal kidney (ki), human adrenal (adr), and human testis (test). lad, Control protein ladder; neg, negative control; cyt b5, cytochrome *b5*; 5α-red-1, 5α-reductase type 1; 5α-red-2, 5α-reductase type 2; 5β-red, 5β-reductase.

20-reduction of 3α,5β-TH-P to 20α-DH,3α,5β-TH-P, but we could not demonstrate 20-oxidation of various other P metabolites (Table 2). The presence of enzymes with major 20α-reductase activity, 20α-HSD (AKR1C1) and 17β-HSD type 5 (AKR1C3), was demonstrated by RT-PCR (Fig. 2).

After incubation of the microsomal fraction from human kidney with radiolabeled P, we detected 5α-DH-P, 20α-DH,5α-DH-P, and 3β,5α-TH-P, indicating 5α-reductase activity. The expression of 5α-reductase type 1 was demonstrated by RT-PCR, whereas 5α-reductase type 2 was not detected (Fig. 2). Generation of 3β,5α-TH-P from P suggests that the kidney also expresses 3β-HSD activity. Consistent

TABLE 2. Summary of detected conversion of different unlabeled steroids in cytosolic and microsomal fraction of human postmenopausal kidneys

Cytosol	
3α,5β-TH-P	→ 20α-DH,3α,5β-TH-P
5α-DH-T	→ 3α-diol
3α-diol	→ Unknown substance
4-dione	→ T
T	→ 5α-DH-T
Microsomes	
4-dione	→ Androstanedione
4-dione	→ T

Experimental conditions are described in *Material and Methods.* DH, Dihydro; TH, tetrahydro; 4-dione, androstenedione; 3α-diol, 3α-androstanediol; P, progesterone; T, testosterone.

with this finding we detected 3β-HSD type 2 mRNA. However, there was no evidence of 3β-HSD type 1 expression (Fig. 2). In contrast to what was shown previously for premenopausal kidney (8), there was no conversion to 3α- or 5β-reduced P metabolites in the postmenopausal kidney tissue used for the current experiments. Despite this lack of functional activity, we still were able to detect the expression of 3α-HSD type 3 (AKR1C2) mRNA and 5β-reductase mRNA in postmenopausal kidney samples (Fig. 2).

In the microsomal fraction we found predominant conversion of P to 17α-OH-P and 17α-OH,20α-DH-P, but also to 16α-OH-P. This suggested the presence of 17α-hydroxylase/17,20-lyase (P450c17), which could be demonstrated by RT-PCR (Fig. 2). Sequencing of the RT-PCR products (exons 1–3) revealed 100% identity to the published P450c17 sequence.

Conversion of Preg and DHEA

The expression and functional activity of P450c17, 3β-HSD type 2, and 5α-reductase type 1 suggested that not only may these enzymes contribute to progesterone metabolism, but that efficient generation of androgens may occur in human kidney. To investigate this in further detail, we incubated both cytosolic and microsomal fractions with radiolabeled Preg or DHEA and in addition with unlabeled T, estrone, 4-dione, 5α-DH-T, or 3α-diol.

In microsomes we detected the conversion of [^{3}H]Preg to 17α-OH-Preg (mean ± SD, 21.6 ± 2.1%) and DHEA (18.0 ± 1.5%), indicating the presence of both 17α-hydroxylase and 17,20-lyase activities of P450c17 (Figs. 1 and 3a). One metabolite peak on the TLC plate could not be identified (mean ± SD, 10.8 ± 1.2% conversion; Fig. 1). In addition, we detected traces of androstenediol in the microsomal fraction, reflecting downstream conversion of DHEA via 17β-HSD activity (Fig. 3a). The 17,20-lyase activity of P450c17, which catalyzes the conversion of 17α-OH-Preg to DHEA, requires allosteric facilitation of the interaction of P450c17 with its electron donor P450 oxidoreductase. This allosteric modulation is mediated by cytochrome *b5* (17, 18), and cytochrome *b5* expression was readily detected by RT-PCR in both human kidney cortex and medulla (Fig. 2).

Microsomal incubations with radiolabeled DHEA and NADPH or NADH as cosubstrate revealed minimal conversion of DHEA to 4-dione (0.5–1%) and androstenediol (0.8–1%), indicating 3β-HSD and 17β-HSD activities (Fig. 3a).

After 120-min incubation of kidney microsomes with 10^{-4} M unlabeled T, we did not detect 5α- or 5β-DH-T. However, there was conversion of 4-dione to T and in small amounts also to androstanedione (Table 2 and Fig. 3a).

In the cytosolic kidney fraction, radiolabeled DHEA was predominantly converted to androstenediol (mean ± SD, 46.9 ± 0.9% conversion), and to two other unknown metabolites (1–3% conversion). These unknown metabolites were not identical to the steroids tested (see *Materials and Methods*). The conversion to androstenediol indicates a strong 17β-HSD activity. In accordance with this, we found efficient conversion of 4-dione to T in human kidney cytosol (Table 2 and Fig. 3b). 17β-HSD activity could be due to all three enzymes of the AKR family (AKR1C1, AKR1C2, and AKR1C3), for which expression was detected in the kidney and which all exhibit 17β-HSD activity. However, 17β-HSD type 5 (AKR1C3) is the most probable enzyme due to its high 17β-HSD activity and widespread cytosolic distribution.

Cytosolic incubations also revealed a small conversion of T to 5α-DH-T, indicating 5α-reductase activity by 5α-reductase type 1 (Table 2 and Fig. 2). 5α-DH-T was inactivated to 3α-diol (Fig. 3b) by 3α-HSD activity.

Discussion

It has long been known that P has potential to interfere with signaling through the MR. This may be relevant to the physiological and pathophysiological changes in fluid balance and blood pressure seen in pregnancy and possibly in the luteal phase of the menstrual cycle or in women taking contraceptives containing synthetic progestins or P itself. P binds *in vitro* with similar affinity to the MR as aldosterone, but confers only weak agonistic MR activity (1–4). This anti-MR potency of P results in increased urinary sodium excretion if given im in large amounts (19–22). During pregnancy, P concentrations exceed those of aldosterone at least 50- to 100-fold. It was not known how aldosterone, with a slight increase in plasma concentration during pregnancy, can act as an MR agonist in situations with high P concentrations. However, P has 10-fold higher plasma protein binding than aldosterone, thereby reducing the unbound steroid fraction of P (23). In addition, P dissociates faster from the MR complex than aldosterone due to a higher stability of the aldosterone-MR complex (2). We believe that a potent and effective P metabolism to inactive metabolites (4) in human kidney could be an equivalent protective mechanism for the protection of the MR similar to the inactivation of cortisol by 11β-HSD type 2 (7, 8). In a previous study we demonstrated that even at high P concentrations the kidney efficiently converted P to downstream metabolites (7). In this study we identified the enzymes responsible for this P metabolism. This is of special interest, because an alteration of P metabolism could be one cause of hypertension and water retention in pregnancy and preeclampsia (7). Here, we used kidney samples from postmenopausal women. However, it should be taken into account that the activation and inactivation of steroid hormones may vary with the sex and age of the donor.

Strong 20α-reduction of P may be due to the catalytic activity of AKR1C1 and/or AKR1C3, which were both expressed in human kidney. It appears that the AKR1C1 (20α-

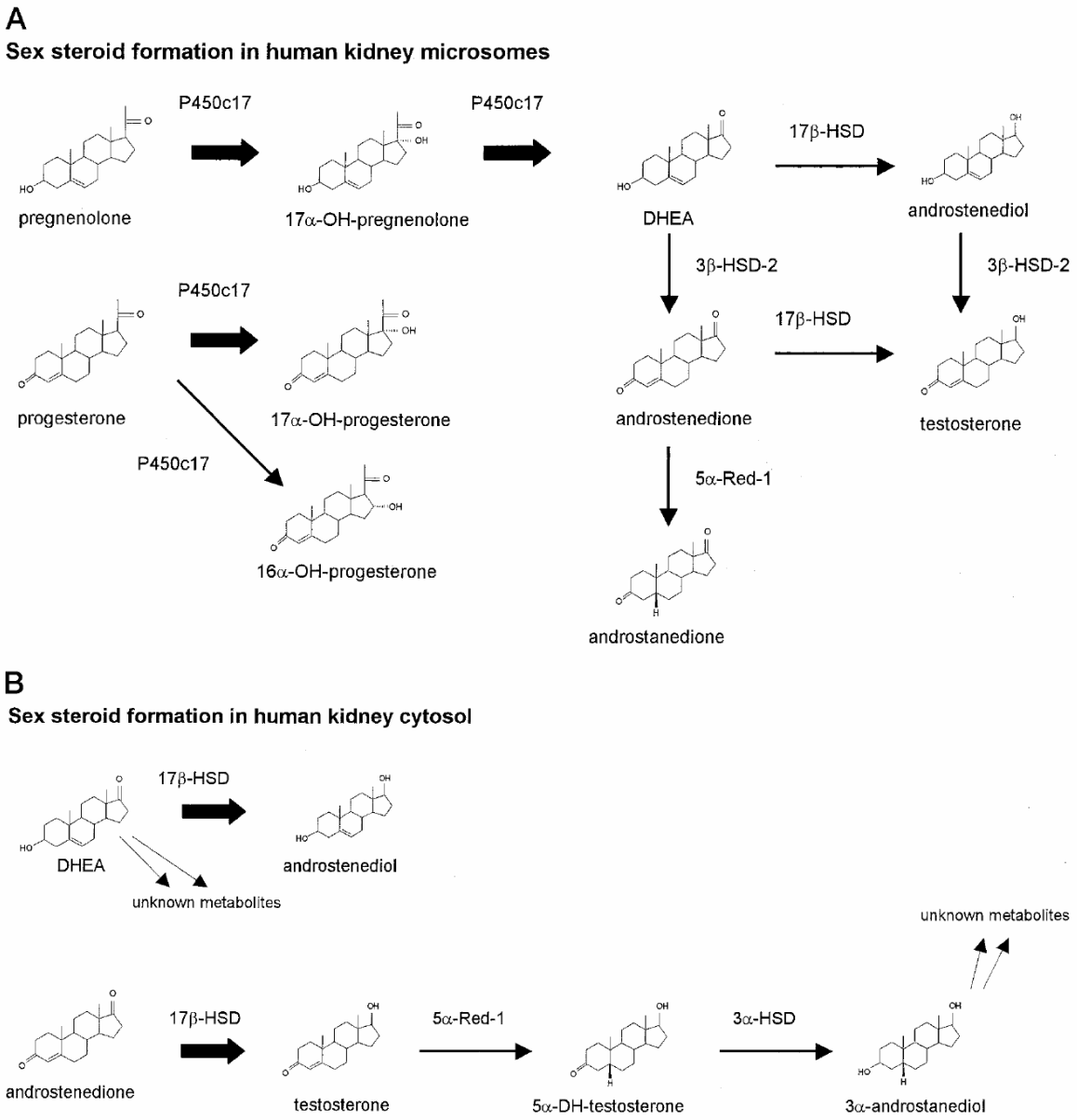

FIG. 3. Schematic representation of sex steroid formation and the involved enzymes in human postmenopausal kidney microsomes (A) and cytosol (B). The thickness of the *arrows* represents the approximate quantity of conversion. Red, Reductase.

HSD) is the most probable enzyme to catalyze 20α-reduction (24). We calculated a K_m for 20α-reduction of P similar to reports in AKR1C1-transfected cells (2.6–18 μmol/liter) (25, 26). However, AKR1C3 (3α-HSD type 2 = 17β-HSD type 5) may also contribute to the conversion of P to 20α-DH-P, because it exerts strong 20α-HSD activity in addition to its 17β-HSD activity (13, 24, 26–28).

We also found efficient 17α-hydroxylation of P in human kidney. The microsomal P450c17 catalyzes 17α-hydroxylation, followed by 17,20-lysis of 17α-hydroxylated products (29), and is expressed in human steroidogenic tissues. Our results are another example of P450c17 expression in extra-adrenal and extragonadal tissues. Besides 17α- and 16α-hydroxylation of P (30–32), we detected the expression of both P450c17 and cytochrome *b5* in human adult kidney, resulting not only in efficient 17α-hydroxylase activity, but also in 17,20-lyase activity of P450c17. Accordingly, we found the conversion of Preg via 17α-OH-Preg to DHEA. Thus, the kidney seems to be the only tissue, except for adrenal, gonad, placenta, and brain, that expresses functionally active

P450c17, and the physiological relevance of this finding needs further investigation.

Further downstream, the conversion of DHEA to 4-dione is catalyzed by 3β-HSD. In human kidney we found the expression of 3β-HSD type 2 converting 5α-DH-P to 3β,5α-TH-P. However, we did not detect the conversion of Preg to P and found only weak conversion of DHEA to 4-dione in human kidney. Therefore, 3β-HSD type 2 seems to play a role in renal steroid metabolism rather than steroidogenesis.

We detected a strong conversion of 4-dione to T (17β-HSD activity) in renal cytosol and therefore assume that this reaction is specific for AKR1C3 (17β-HSD type 5) because we could not detect 17β-HSD type 3 mRNA in human kidney. We also demonstrated 5α-reductase type 1 expression in human kidney tissue, which was snap-frozen immediately. In contrast to our finding, Thigpen *et al.* (33) were not able not detect 5α-reductase type 1 mRNA; however, they used postmortem kidneys. We found functional activity of 5α-reductase type 1 resulting in 5α-reduction of P to 5α-DH-P, of 4-dione to androstanedione, and of T to the potent androgen 5α-DH-T. Further studies are necessary to investigate the local production of 5α-DH-T within the kidney, its possible influence on water and salt retention, and its impact on the pathogenesis of hypertension. In addition, 5α-metabolites of P exert various biological functions, such as anxiolytic properties, relaxing potency of smooth muscle cells, and neuroactive γ-aminobutyric acid A receptor agonistic properties, *e.g.* 3α,5α-TH-P (34–37). Thus, 5α-reduction could also represent an important modulatory activity with regard to P metabolism.

We suggest that the described P metabolism in MR target cells is a possible protection mechanism for the MR. Therefore, these enzymes may be relevant to physiological and pathophysiological changes in fluid balance and blood pressure. An alteration of expression of these enzymes may play a role in pregnancy-induced hypertension, preeclampsia, and the premenstrual syndrome.

In vitro, P has a positive effect on the relaxation of smooth muscle cells within the uterus as well as in extrauterine tissue (38). Therefore, it may be possible that P regulates renal blood flow, reducing the renal effect of angiotensin II and increasing the glomerular filtration rate (39). The underlying mechanism is still unknown, but P-metabolizing enzymes may play a regulatory role in renal blood flow.

In addition, it was recently shown that P enhances calcium reabsorption in distal tubules and collecting duct of rabbit kidney (40), probably mediated by the P receptor, which is expressed in the kidney (40). Renal P-metabolizing enzymes may regulate the access of P to the P receptor and consequently alter the effect of P on calcium homeostasis.

In postmenopausal women all sex steroids are of extragonadal origin. Several tissues produce androgens and estrogens from precursor steroids secreted by the adrenals (*e.g.* DHEA and DHEA sulfate). We investigated the synthesis and metabolism of androgenic steroids in human postmenopausal kidneys and were able to characterize in detail a complex steroid enzymology in the human kidney. The kidney is able to synthesize DHEA from Preg via 17α-OH-Preg, with further downstream activation via 4-dione to T and 5α-DH-T. The physiological importance of androgen generation in the kidney remains to be elucidated. Androgens appear to play an important role in the pathogenesis of hypertension and in modulating sex-specific differences in hypertension. This hypothesis is supported by the finding that castration of spontaneously hypertensive male rats or treatment with the androgen receptor antagonist flutamide lowers blood pressure and slows the progression of hypertensive organ damage to the kidney (41). Administration of T to these castrated rats or to female rats resulted in increases in blood pressure to the level in untreated male rats, and renal injury subsequently showed fast progression. This effect could be mediated directly via activation of the renin-angiotensin system or via increased proximal tubular reabsorption (41–43).

In conclusion, we clearly established the effective generation of androgens in the human kidney by locally expressed steroidogenic enzymes, and it remains an important goal of future research to understand the physiological role of androgen formation in the kidney.

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