3. Relevante Originalarbeiten

Im Folgenden sind die wichtigsten, relevanten Originalarbeiten aufgeführt.

3.1 11β-Hydroxysteroid Dehydrogenase (11β-HSD)

3.1.1 11β-HSD Isoenzyme beim Meerschweinchen

1. **Quinkler M**, Kosmale B, Bähr V, Oelkers W, Diederich S.
   Evidence for isoforms of 11β-hydroxysteroid dehydrogenase in liver and kidney of the guinea pig.
   J.Endocrinol. **1997**, 153(2): 291-298.

Evidence for isoforms of 11β-hydroxysteroid dehydrogenase in the liver and kidney of the guinea pig

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Abstract

In the human and in rodents like the rat and mouse, the liver enzyme 11β-hydroxysteroid dehydrogenase type I (11β-HSD-I) is a functional oxidoreductase preferring $NADP^+$/NADPH as cosubstrate, while the renal isoenzyme (11β-HSD-II) prefers NAD^+ as cosubstrate, and seems to be a pure oxidase and protects the tubular mineralocorticoid (MC) receptor from occupancy by cortisol and corticosterone. We studied the enzyme kinetics of 11β-HSDs in kidney and liver microsomes of the guinea pig, a species whose zoological classification is still a matter of debate. With a fixed concentration of 10^{-6} mol/l cortisol, liver and kidney microsomes preferred NAD^+ to $NADP^+$ (10^{-3} mol/l) for the conversion to cortisone. Kidney microsomes converted cortisol to cortisone with K_m values of 0·64 μmol/l and 9·8 μmol/l with NAD^+ and $NADP^+$ as cosubstrates respectively. The reduction of cortisone to cortisol was slow with kidney microsomes, but could be markedly enhanced by adding an NADH/NADPH regenerating system: with NADPH as preferred cosubstrate, the approximate K_m was 7·2 μmol/l. This indicated the existence of both isoenzymes in the guinea pig kidney. Liver microsomes oxidized cortisol to cortisone with similar K_m and V_{max} values for NAD^+ to $NADP^+$ as cosubstrates (K_m of 4·3 μmol/l and 5·0 μmol/l respectively). The NAD^+ preference for the oxidation of 10^{-6} mol/l cortisol described above may be due to a second, NAD^+-preferring 11β-HSD with a K_m of 1·4 μmol/l. In contrast to the kidney, liver microsomes actively converted cortisone to cortisol with a preference for NADPH (K_m: 1·2 μmol/l; V_{max}: 467 nmol/min per mg protein). Thus, the main liver enzyme is similar to the oxidoreductase of other species (11β-HSD-I) and is also present in the kidney, while the main kidney enzyme is clearly NAD^+-preferring. This kidney enzyme (analogous to 11β-HSD-II of other species) seems to be suitable for the protection of the MC receptor from the high free plasma cortisol levels of the guinea pig.

Journal of Endocrinology (1997) **153,** 291–298

Introduction

11β-Hydroxysteroid oxidoreductases (11β-HSDs) convert 11-hydroxysteroids like cortisol into 11-oxosteroids like the hormonally inactive cortisone and vice versa. In both man and rat, the long-known 11β-HSD of the liver (isoenzyme I) is active in both the oxidative and reductive direction, has a relatively high Michaelis–Menten constant (K_m) in the micromolar range and prefers $NADP^+$/NADPH as cosubstrates. It is also present in many other tissues and may modulate the access of cortisol and corticosterone to the glucocorticoid (GC) receptor (Edwards *et al.* 1988, Funder *et al.* 1988).

An isoenzyme (11β-HSD-II) that is present in the placenta and in tissues harbouring the mineralocorticoid (MC) receptor (kidney, colon, salivary glands) has recently been discovered in the human and the rat. The MC receptor has similar affinity for aldosterone, corticosterone and cortisol (Arriza *et al.* 1987). In the kidney and the colonic mucosa, 11β-HSD-II with a K_m in the nanomolar range greatly reduces the intracellular cortisol concentration and allows aldosterone, which is not a substrate of the enzyme, to bind selectively to the MC receptor (Stewart *et al.* 1994). The enzyme is believed to function exclusively as an oxidase (Albiston *et al.* 1994), but with 9α-fluorinated 11-oxo-steroids as substrates, it is a strong reductase in the human (Oelkers *et al.* 1994, Diederich *et al.* 1996*a*). 11β-HSD-II uses NAD^+/(NADH) as cosubstrates exclusively. In the human kidney, predominantly 11β-HSD-II seems to be present (Tannin *et al.* 1991, Whorwood *et al.* 1995), while in rat and mouse kidneys, both isoenzymes could be clearly demonstrated (Agarwal *et al.* 1989, Rajan *et al.* 1995).

The guinea pig (*Cavia porcellus*) probably belongs to the New World subgroup of rodents called hystricomorphs. However, the question as to whether the guinea pig should be allocated to a separate mammalian order has not yet been resolved (Novacek 1992). There are numerous anomalies in the guinea pig endocrine system, as recently reviewed by Keightley & Fuller (1996). With regard to

Journal of Endocrinology (1997) **153,** 291–298 *Printed in Great Britain*
0022–0795/97/0153–0291 $08.00/0

the pituitary-adrenal axis, guinea pig adrenocorticotrophin has increased biopotency (Smith *et al.* 1987), plasma levels of cortisol are very high (Claman 1972) and the affinity of cortisol to cortisol-binding globulin (CBG) is weak (Westphal 1967), while its affinity to the GC receptor is low (Kraft *et al.* 1979). The MC receptor of the guinea pig, however, shows no difference from that of other species (Myles & Funder 1994). Since free plasma cortisol levels are much higher in the guinea pig than in the human, while plasma aldosterone is in the same range in both species (Whipp *et al.* 1976), it seems reasonable to assume that a highly efficient mechanism exists to exclude cortisol from the MC receptors in the guinea pig. One of these mechanisms could be 11β-HSD-II with a high activity in MC target tissues (Keightley & Fuller 1996), as in the human.

Only the 11β-HSD-I in the guinea pig kidney has been studied up to now (Sang *et al.* 1991, Zhang *et al.* 1994, Lee *et al.* 1996). The 11β-HSD-II isoenzyme has not been described in this species, and data on guinea pig liver 11β-HSD are not yet available in the literature. In this paper, we present data on 11β-HSD in guinea pig liver and kidney microsomes and characterize the isoenzymes.

Materials and Methods

Chemicals and solutions

Cortisol and cortisone were obtained from Sigma Chemical Co. (St Louis, MO, USA), [1,2,6,7-^{3}H(N)]cortisol (specific activity: 70·0 Ci/mmol) from DuPont de Nemours GmbH (Bad Homburg, Germany), and [1,2(n)-^{3}H]cortisone (specific activity: 41 Ci/mmol) from Amersham International plc (Amersham, Bucks, UK). Both tritiated steroids were purified by HPLC prior to use. The steroids were dissolved in methanol and kept at −20 °C. Sodium-hydrogenphosphate-dihydrate and sodium-hydrogenphosphate-monohydrate were from Merck Ltd (Darmstadt, Germany), sucrose was from Sigma Chemical Co. and NaCl solution (0·9 g/l) was from Braun Melsungen Ltd (Melsungen, Germany). The cofactors β-NADPH-tetrasodium salt, β-NADH-disodium salt, β-NADP$^+$-monosodium salt and β-NAD$^+$ and D-glucose-6-phosphate-monosodium salt were purchased from Sigma Chemical Co. Glucose-6-phosphate-dehydrogenase (from *Leuconostoc mesenteroides*) in 3·2 mol/l $(NH_4)_2SO_4$, 50 mmol/l Tris and 1 mmol/l $MgCl_2$, pH 7·5 solution was obtained from Boehringer (Mannheim, Germany). We used the following solvents for sample clean-up and chromatography: acetone, n-hexane and 2-propanol at LiChrosolv quality, obtained from Merck Ltd, and methanol at LiChrosolv quality from J T Baker BV (Deventer, The Netherlands). 20α-Dihydroxy-cortisone (20α-DH-E), 20β-DH-E and 6β-hydroxy-cortisone (6β-OH-E) were obtained from Sigma Chemical Co.

Enzyme preparation and measurement of 11β-HSD activity

Kidney cortex and liver tissues were obtained from female haired Duncan–Hartley guinea pigs. Tissues were homogenized in sodium phosphate buffer, containing 0·25 mol/l sucrose. All subsequent steps were performed at 0–4 °C. Microsomes were prepared as described previously (Monder & Lakshmi 1989) by centrifugation at 750 ***g*** for 30 min and at 20 000 ***g*** for 30 min saving the supernatant at each step. The last supernatant was recentrifuged at 105 000 ***g*** for 60 min, and the pellet was resuspended and recentrifuged at 105 000 ***g*** for 60 min. Aliquots containing the microsomes were stored in liquid nitrogen. Microsomes were used within 2 months, except aliquots for characterizing the reductase reaction by liver microsomes, and no loss in enzyme activity was seen during this period (data not shown). Protein quantification was done before incubations with the method described by Lowry *et al.* (1951), and the microsomes were diluted to the concentrations required.

Incubations were carried out in wells of a plastic multiwell incubation plate located in a steel chamber that had been preheated to 37 °C in a water bath under continuous shaking. The total incubation volume was 1 ml, consisting of 790 µl sodium phosphate buffer, 100 µl of a 10^{-2} molar cofactor solution (NAD$^+$, NADP$^+$, NADH, NADPH), 100 µl microsome supension and 10 µl [^{3}H]cortisol or [^{3}H]cortisone (each 90 000 c.p.m.) and unlabelled cortisol or cortisone in concentrations varying from 10^{-8} mol/l to 10^{-5} mol/l in methanol solution. Methanol concentration was kept at $\leq 1\%$.

Based on the results of Lakshmi *et al.* (1993), we used a sodium phosphate buffer of pH 8·5 for the dehydrogenase reaction, and the same buffer system with pH 6·0 for the reductase reaction. Since spontaneous reduction of the 11-oxo-group of cortisone by kidney microsomes was too slow for performing kinetic analyses, especially with NADH, we added 10^{-2} mol/l D-glucose-6-phosphate and 10 units glucose-6-phosphate-dehydrogenase as a NADH/NADPH regenerating system (Agarwal *et al.* 1990). Figure 1 shows the effect of this system on the percentage reduction with NADH as cosubstrate. With NADPH as cosubstrate, the results were comparable, but conversion (%) was higher than with NADH as cosubstrate. The cofactors were used in an excess concentration of 10^{-3} mol/l (Lakshmi *et al.* 1993, Albiston *et al.* 1994). In preliminary studies we determined the optimal conditions for obtaining an initial linear reaction velocity by varying the amount of enzyme and the reaction time. A concentration of 0·1–0·3 mg protein/ml was used. Blanks were included containing all assay components except the enzyme that was replaced by buffer in all assays. All data reported represent means of three or five independent incubations.

Incubations were started by the addition of microsomes to wells preincubated for 10 min containing all the assay

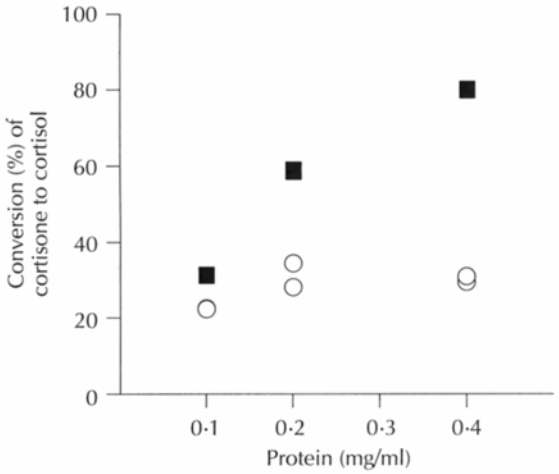

Figure 1 Conversion (%) of cortisone to cortisol with NADH as cosubstrate (10^{-3} mol/l) in guinea pig kidney microsomes with (■) or without (○) using a NADH/NADPH regenerating system (10^{-2} mol/l glucose-6-phosphate and 10 units glucose-6-phosphate-dehydrogenase) and varying protein concentrations. For each protein concentration $n=2$ with or without the regenerating system. 120 min incubation time, $3{\cdot}16 \times 10^{-8}$ mol/l cortisone and pH 6·0. With NADPH as cosubstrate, results were comparable, but conversion (%) was higher (with and without the regenerating system).

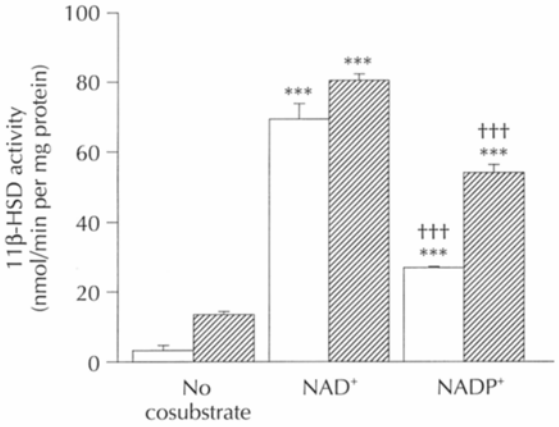

Figure 2 Characterization of cosubstrate specificity for the oxidation of cortisol (10^{-6} mol/l) to cortisone by guinea pig kidney (open bars) and liver (hatched bars) microsomes. Cosubstrate concentration: 10^{-3} mol/l; pH 8·5. Values are means ± S.D. of five experiments. ***$P<0{\cdot}001$ compared with control (no cosubstrate), and †††$P<0{\cdot}001$ compared with NAD^+.

components except the enzyme preparation. Incubations were terminated by the addition of 2 ml cold methanol and rapid transfer of the incubation plate into an ice-bath. The media were centrifuged at 3000 U/min for 10 min at 4 °C, the supernatant was transferred into plastic tubes, 6 ml H_2O were added and each sample was extracted by Sep-Pak cartridges C_{18} (Waters Millipore GmbH, Eschborn, Germany) as described previously (Eckhoff *et al.* 1988). Samples were kept at − 20 °C until the analytic procedure. They were injected into a normal-phase HPLC column running with an isocratic solvent mixture (80% hexane and 20% 2-propanol, flow-rate 1·3 ml/min) for 12 min. The steroids were located on the chromatogram and quantified by UV detection and by ^3H detection using a radioactivity monitor, as described previously (Diederich *et al.* 1996*b*). The retention times were 6·4 min for cortisone and 8·2 min for cortisol. The sum of the two peaks was defined as 100%, and the percentage of the newly formed metabolite is reported.

Preliminary experiments with liver microsomes of the guinea pig with cortisone as substrate and with NADH as cosubstrate showed a small broad-based ^3H-labelled steroid peak in addition to the newly formed [^3H]cortisol, which probably consisted of 20α-DH-E, 20β-DH-E and/or 6β-OH-E (additional HPLC UV detection with 210 nm wave length and 20 min run-time). Kinetic data for the conversion of cortisone to cortisol with NADH in the liver are, therefore, of an approximate nature. All other experiments with guinea pig microsomes did not show any conversion into metabolites other than cortisol and cortisone.

Statistical calculations were performed using an SPSS program from SPSS Inc. (Chicago, IL, USA) and an MS-Excel program 5·0 from Microsoft (Microsoft Corporation, USA). The Duncan multiple range test and *t*-test were employed.

For the calculation of K_m and maximal reaction velocities (V_{max}) values we used the Eadie–Hofstee transformation (Dowd & Riggs 1965). In addition, intrinsic clearance values ($CL_{int}=V_{max}/K_m$) are reported. This term describes originally the metabolism rate of a drug under *in vivo* conditions, and can be used as a parameter for the *in vivo* importance of *in vitro* experiments if linear Michaelis–Menten conditions are given (Houston 1994).

Results

Cofactor preference

Kidney In kidney micosomes, the preferred cofactor for the oxidase reaction was NAD^+ (Fig. 2) as in the liver. Activities in the presence of NAD^+ and $NADP^+$ were 69 ± 5 and 27 ± 0·2 nmol/min per mg protein respectively. 11-Keto reduction without the NADH/NADPH regenerating system was slow using kidney microsomes (Fig. 1). NADPH was preferred for the reductase reaction in the kidney in the presence of an NADH/NADPH regenerating system (Fig. 3). With this system, the activities in the presence of NADH and NADPH for

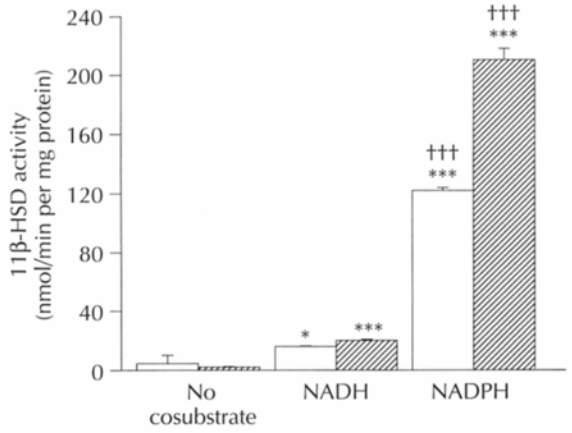

Figure 3 Characterization of cosubstrate specificity for the reduction of cortisone (10^{-6} mol/l) to cortisol by guinea pig kidney (open bars) and liver (hatched bars) microsomes. In kidney microsomes an NADH/NADPH regenerating system (10^{-2} mol/l glucose-6-phosphate and 10 units glucose-6-phosphate-dehydrogenase) was used. Cosubstrate concentration: 10^{-3} mol/l; pH 6·0. Values are mean ± S.D. of three experiments. *P<0·05 and ***P<0·001 compared with control (no cosubstrate), and †††P<0·001 compared with NADH.

the conversion of cortisone to cortisol were 16 ± 0·4 and 122 ± 2 nmol/min per mg protein respectively (Fig. 3).

Liver At variance with findings in the human and the rat, liver microsomes from the guinea pig oxidized cortisol with a slightly higher activity with NAD^+ than with $NADP^+$ as cosubstrate (Fig. 2). Activities in the presence of NAD^+ and $NADP^+$ were 80 ± 2 and 54 ± 2 nmol/min per mg protein respectively. For the 11-keto reduction of cortisone by liver microsomes (without the NADH/NADPH regenerating system) NADPH was the preferred cosubstrate with an activity of 211 ± 8 nmol/min per mg protein (Fig. 3). Activity in the presence of NADH was much lower (20 ± 1 nmol/min per mg protein).

Kinetic data

Kidney With cortisol as the physiological substrate, the K_m for cortisol with $NADP^+$ was 9·8 ± 0·4 μmol/l, and V_{max} was 298 ± 10 nmol/min per mg protein. With NAD^+ as cosubstrate the K_m was 0·64 ± 0·03 μmol/l and V_{max} was 124 ± 4 nmol/min per mg protein (Fig. 4*c* and Table 1). For this reaction, the calculated CL_{int} values were much higher with NAD^+ than with $NADP^+$ as cosubstrate (Table 1).

For the 11-keto-reduction of cortisone in the kidney in the presence of the NADH/NADPH regenerating system, K_m in the presence of NADH was 7·2 ± 0·5 μmol/l and V_{max} was 146 ± 10 nmol/min per mg protein. With NADPH as cosubstrate, K_m (3·1 ± 0·2 μmol/l) was similar, but V_{max} was higher (440 ± 27 nmol/min per mg protein) (Table 1). Thus, CL_{int} values calculated for the reductase reaction with NADH were much lower than those with NADPH as cosubstrate.

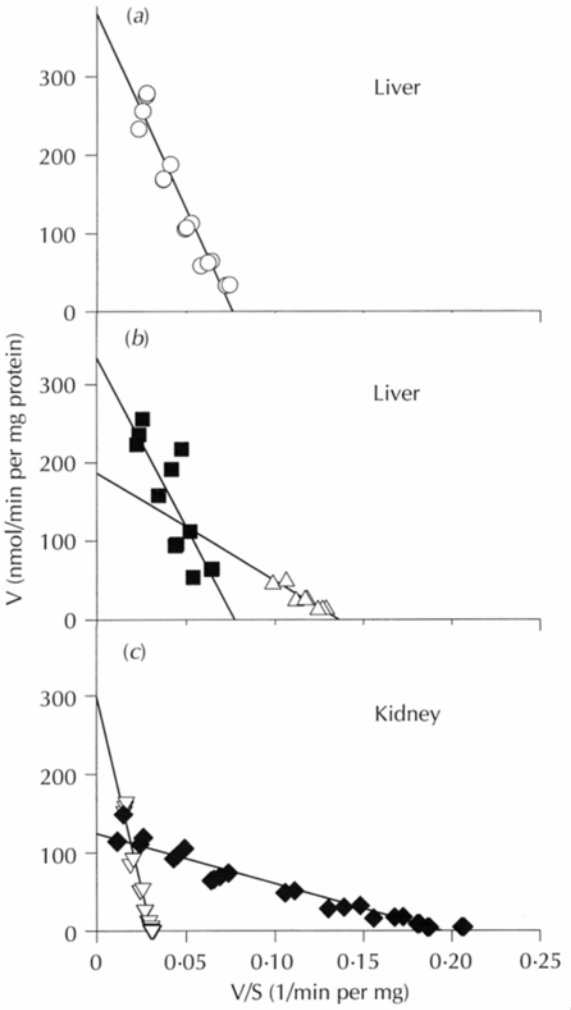

Figure 4 Enzyme kinetics of the oxidation of cortisol to cortisone by guinea pig microsomes. Cosubstrate concentration was always 10^{-3} mol/l. Data are presented as Eadie–Hofstee plots. Steroid concentrations ranged from $2·14 \times 10^{-8}$ mol/l to 10^{-5} mol/l. Each point represents one incubation. (*a*) Liver microsomes: ○=HSD with $NADP^+$, correlation r^2=0·93; (*b*) liver microsomes: ■=HSD with NAD^+, r^2=0·70 and △=HSD with NAD^+, r^2=0·85; (*c*) kidney microsomes: ▽=HSD with $NADP^+$, r^2=0·96 and ◆=HSD with NAD^+, r^2=0·95.

Liver For the oxidation of cortisol to cortisone, NAD^+ and $NADP^+$ were used by liver microsomes with similar

Table 1 Kinetic parameters for guinea pig 11β-HSD activity of liver and kidney microsomes (F=cortisol; E=cortisone). (*)=glucose-6-phosphate (10^{-2} mol/l) and glucose-6-phosphate-dehydrogenase (10 units) were added as an NADH/NADPH regenerating system. The reductase reaction with NADH in liver microsomes produced an additional ^{3}H peak, probably consisting of cortisone metabolites (20α-DH-E, 20β-DH-E, 6β-OH-E), which made up less than 10% of the total radioactivity. Kinetic data for the reductase reaction with NADH in liver microsomes are therefore approximate values. CL_{int}=intrinsic clearance values (V_{max}/K_m). Values are means ± S.D.

	Liver				
Reaction:		F→E		E→F	
		HSD-I		HSD-I	
Cofactor:	HSD-? NAD^+	NAD^+	$NADP^+$	NADH	NADPH
K_m (μmol/l)	1·4 ± 0·2	4·3 ± 0·9	5·0 ± 0·4	4·8 ± 0·5	1·2 ± 0·1
V_{max} (nmol/min per mg)	186 ± 25	334 ± 40	380 ± 18	135 ± 12	467 ± 15
CL_{int} (ml/mg per min)	136	77	76	28	406
	Kidney				
Reaction:		F→E		E→F	
				HSD-I	
		HSD-II	HSD-I		
Cofactor:		NAD^+	$NADP^+$	NADH(*)	NADPH(*)
K_m (μmol/l)		0·64 ± 0·03	9·8 ± 0·4	7·2 ± 0·5	3·1 ± 0·2
V_{max} (nmol/min per mg)		124 ± 4	298 ± 10	146 ± 10	440 ± 27
CL_{int} (ml/mg per min)		194	30	20	143

K_m values in the micromolar range (4·3 ± 0·9 and 5·0 ± 0·4 μmol/l respectively) and with similar maximal velocities (Fig. 4*a* and *b* and Table 1). However, the results plotted in Fig. 4*b* showing a discrete straight line for low cortisol concentrations with NAD^+ as cosubstrate can be interpreted by assuming the presence of a second NAD^+-preferring 11β-HSD (K_m: 1·4 ± 0·2 μmol/l; V_{max}: 186 ± 25 nmol/min per mg protein) in the liver.

Reduction of cortisone to cortisol by liver microsomes in the presence of NADPH was very active (Fig. 5) (K_m: 1·2 ± 0·1 μmol/l; V_{max}: 467 ± 15 nmol/min per mg protein). This is also illustrated by a very high CL_{int} value (Table 1). The reductase reaction with NADH as cosubstrate could be quantitated only approximately since other metabolites of cortisone (<10% of total radioactivity) were formed (see Materials and Methods section). However, it is clear that the K_m with NADH as cosubstrate (approximately 4·8 ± 0·5 μmol/l) was higher and the V_{max} (approximately 135 ± 12 nmol/min per mg protein) lower than with NADPH, resulting in a much lower CL_{int} value.

Discussion

In humans about 90% of plasma cortisol is bound to CBG and to albumin (7%), and only 4% (6–16 nmol/l) is unbound (Dunn *et al.* 1981). In guinea pigs, cortisol is the major glucocorticoid, as in man, but total plasma cortisol levels are higher than in man (Fujieda *et al.* 1982, Redmann *et al.* 1995) and CBG has a 10- to 20-fold (Westphal 1967, Fujieda *et al.* 1982) lower affinity to cortisol than in the human. Therefore, free plasma cortisol levels are at least three times higher (20–160 nmol/l) in the guinea pig (Fujieda *et al.* 1982). Since the MC receptor of the guinea pig has the same affinity for aldosterone and cortisol as in the human (Myles & Funder 1994), the guinea pig requires highly effective mechanisms for the exclusion of cortisol from this receptor.

Enzyme kinetics of guinea pig 11β-HSD were previously studied by Sang *et al.* (1991), Zhang *et al.* (1994) and Lee *et al.* (1996) in kidney microsomes and with $NADP^+$ as cosubstrate. These authors found K_m values for the oxidase reaction of 2·7 μmol/l (substrate: corticosterone), 8 μmol/l (cortisol) and 57·6 μmol/l (cortisol). In the study of Lee *et al.* (1996) the K_m for cortisol with NAD^+ was also very high (36·4 μmol/l). With regard to the properties of 11β-HSD isoenzymes, the authors cited above probably described 11β-HSD-I in the guinea pig kidney and not the 11β-HSD isoenzyme (type II), which protects the MC receptor. The above authors used male guinea pigs, whereas we used females. Since sex differences in 11β-HSD expression (Low *et al.* 1994*b*, Albiston *et al.* 1995, Rajan *et al.* 1995) have been well described, the comparison of the results must take possible sex differences into account.

11β-HSD of the kidney

With regard to our results in renal microsomes, where NAD^+ was the preferred cofactor (Fig. 2), we most probably identified two isoenzymes. One isoenzyme with low affinity is bidirectional and prefers NADPH for the reductase reaction (Table 1). The properties of the dehydrogenase reaction with $NADP^+$ as cofactor (K_m: 9·8 μmol/l; V_{max}: approximately 300 nmol/min per mg protein) were similar to those found by Zhang *et al.* (1994) (K_m: 8·0 μmol/l; V_{max}: approximately 500 nmol/min per mg protein). The characteristics of this reaction with $NADP^+$ as cofactor (K_m, V_{max} and CL_{int} values) were, furthermore, similar to our findings in liver microsomes with this cofactor. Since we had to use the NADH/NADPH regenerating system for the kinetic description of the reduction of cortisone by kidney microsomes, comparison of the V_{max} and CL_{int} values for this reaction in the two organs is not permissible. However, the cosubstrate preference and the K_m values are comparable with those found in the liver (Table 1) and similar to those described for 11β-HSD-I in other species (Jamieson *et al.* 1995, Maser & Bannenberg 1994*a*,*b*). Using the NADH regenerating system, we described 11β-HSD-II reduction of cortisone in human kidney microsomes (Diederich *et al.* 1996*a*). However, the characteristics of this reduction (NADH as clearly preferred cosubstrate, K_m in the nanomolar range and a very low V_{max}) are different from those found in the guinea pig experiments.

Thus, our findings suggest that we characterized oxidation of cortisol and reduction of cortisone by 11β-HSD-I in guinea pig kidney microsomes. The 11β-HSD-I expression in the kidney is species-dependent with high levels in rat and mouse (Agarwal *et al.* 1989, Rajan *et al.* 1995) and very low levels in the human (Tannin *et al.* 1991, Whorwood *et al.* 1995).

The characteristics of the second isoenzyme in the kidney are in agreement with 11β-HSD-II of other species: high affinity for cortisol (approximately 15-fold lower K_m values than 11β-HSD-I) and a clearcut NAD^+ preference. Guinea pig 11β-HSD-II has a lower affinity for cortisol (K_m of 0·64 ± 0·03 μmol/l) as compared with other species, but a higher CL_{int} value than in other species (Maser & Bannenberg 1994*b*, Diederich *et al.* 1996*a*). Although the apparent K_m value is significantly higher than free plasma levels of cortisol in the guinea pig (20–160 nmol/l), we propose that this isoenzyme protects the MC receptor from high levels of cortisol, because of its high CL_{int} value.

11β-HSD of the liver

Our kinetic studies with female guinea pig liver microsomes show similar K_m, V_{max} and CL_{int} values for the oxidation of cortisol with NAD^+ or $NADP^+$ as cosubstrates (Table 1). In this regard the liver enzyme of the guinea pig resembles that of the mouse which also accepts the two cosubstrates in a similar way (Maser & Bannenberg 1994*a*,*b*). 11β-HSD-I of the liver functions predominately as a reductase (Low *et al.* 1994*a*, Jamieson *et al.* 1995) and prefers NADPH as cosubstrate (Maser & Bannenberg 1994*a*). This is consistent with our findings in liver microsomes, which exhibit strong NADPH-preferring reductase activity in the absence of the NADH/NADPH regenerating system (Fig. 5). The CL_{int} value of the reductase reaction (406 ml/mg per min) was higher than that of any other reaction (Table 1).

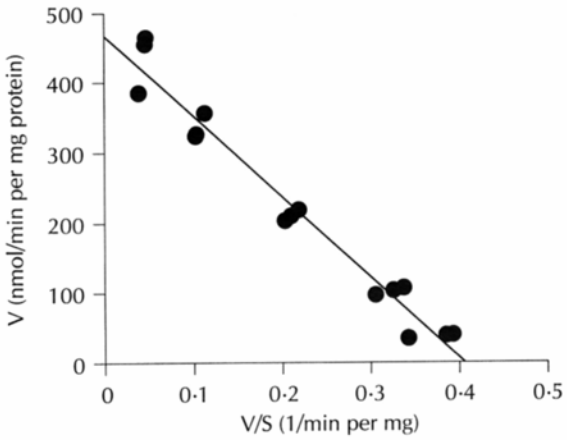

Figure 5 Enzyme kinetics of the reduction of cortisone to cortisol by 11β-HSD in guinea pig liver microsomes with NADPH (10^{-3} mol/l) as cosubstrate. Steroid concentrations ranged from 10^{-8} mol/l to 10^{-5} mol/l. Each point represents one incubation. Data are presented as an Eadie–Hofstee plot. Correlation r^2=0·96.

The results presented in Fig. 4*b* and Table 1 can be interpreted as follows: the guinea pig liver contains a second 11β-HSD with a preference for NAD^+ as cofactor and a K_m of 1·4 ± 0·2 μmol/l for cortisol. This additional enzyme could have caused the NAD^+ preference in the oxidation of 10^{-6} mol/l cortisol shown in Fig. 2. The second, NAD^+-preferring enzyme seems to exhibit a high CL_{int} value similar to that of the renal NAD^+-preferring 11β-HSD-II. Whether this enzyme represents the HSD-II of the liver or a third HSD isoenzyme remains to be elucidated. An additional 11β-oxidizing enzyme in the guinea pig liver could be a physiological explanation for the lack of many other cortisol-metabolizing reactions in guinea pig liver microsomes compared with other species (Abel *et al.* 1993).

In conclusion, we have produced evidence for the existence of 11β-HSD-II and -I in the guinea pig kidney and of 11β-HSD-I in the liver. Because of its high instrinsic clearance value, 11β-HSD-II of the kidney may represent an effective mechanism for protecting the MC receptor from high free cortisol levels in this species. The guinea pig liver may, furthermore, contain some

11β-HSD-II or a third isoenzyme, but this finding needs to be scrutinized by other methods.

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Received 7 May 1996
Revised manuscript received 12 November 1996
Accepted 2 January 1997

3.1.2 Regulation der 11β-HSD durch Stress

2. **Quinkler M**, Troeger H, Eigendorff E, Maser-Gluth C, Stiglic A, Oelkers W, Bähr V, Diederich S.
   Enhanced 11β-hydroxysteroid dehydrogenase type 1 activity in stress adaptation in the guinea pig.
   J.Endocrinol. **2003,** 176(2): 185-192.

Enhanced 11β-hydroxysteroid dehydrogenase type 1 activity in stress adaptation in the guinea pig

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Abstract

The 11β-hydroxysteroid dehydrogenases (11β-HSDs) convert cortisol to its inactive metabolite cortisone and vice versa. 11β-HSD type 1 (11β-HSD-1) functions as a reductase *in vivo*, regulating intracellular cortisol levels and its access to the glucocorticoid receptor. In contrast, 11β-HSD-2 only mediates oxidation of natural glucocorticoids, and protects the mineralocorticoid receptor from high cortisol concentrations. We investigated the *in vivo* and *in vitro* effects of ACTH on the recently characterized 11β-HSDs in guinea pig liver and kidney.

Tissue slices of untreated guinea pigs were incubated with ^{3}H-labelled cortisol or cortisone and $ACTH_{1-24}$ (10^{-10} and 10^{-9} mol/l). The 11β-HSD activities in liver and kidney slices were not influenced by *in vitro* incubation with $ACTH_{1-24}$.

In addition, guinea pigs were treated with $ACTH_{1-24}$ or saline injections s.c. for 3 days. Liver and kidney tissue slices of these animals were incubated with ^{3}H-labelled cortisol or cortisone. *In vivo* ACTH treatment significantly increased reductase and decreased oxidase activity in liver and kidney. Furthermore, 11β-HSD-1 activity assessed by measurement of the urinary ratio of (tetrahydrocortisol (THF)+5αTHF)/(tetrahydrocortisone) was significantly increased after ACTH treatment compared with the control group. Plasma levels of cortisol, cortisone, progesterone, 17-hydroxyprogesterone and androstenedione increased significantly following *in vivo* ACTH treatment.

The enhanced reductase activity of the hepatic and renal 11β-HSD-1 is apparently caused by cortisol or other ACTH-dependent steroids rather than by ACTH itself. This may be an important fine regulation of the glucocorticoid tonus for stress adaptation in every organ, e.g. enhanced gluconeogenesis in liver.

Journal of Endocrinology (2003) **176,** 185–192

Introduction

The 11β-hydroxysteroid dehydrogenases (11β-HSDs) convert 11-hydroxysteroids like cortisol and corticosterone to their inactive metabolites cortisone and 11-dehydrocorticosterone and vice versa. Two different isoenzymes of 11β-HSD have been cloned in the human (Tannin *et al.* 1991, Albiston *et al.* 1994) and several other animal species. The NAD^{+}/(H)-dependent 11β-HSD type 2 (11β-HSD-2) enzyme is found in the placenta and in cells expressing the mineralocorticoid (MC) receptor (Albiston *et al.* 1994, Stewart 1996, Stewart & Krozowski 1999, Quinkler *et al.* 2001). With the endogenous glucocorticoids (GCs) cortisol and corticosterone as substrates, 11β-HSD-2 acts exclusively as a dehydrogenase, whereas with 9α-fluorinated steroids as substrates it is mainly a reductase (Oelkers *et al.* 1994, Diederich *et al.* 1997, Li *et al.* 1997). Further characteristics of 11β-HSD-2 are the strong end-product inhibition by 11-dehydro-GCs (Rusvai & Náray-Fejes-Tóth 1993) and the ability to oxidize dexamethasone (Best *et al.* 1997, Li *et al.* 1997). The main function of 11β-HSD-2 is the protection of the unselective MC receptor, which has similar affinity to cortisol and aldosterone (Arriza *et al.* 1987). This isoenzyme converts large amounts of cortisol to cortisone, thus allowing the lower concentrated aldosterone to bind to the MC receptor (Edwards *et al.* 1988, Funder *et al.* 1988, Stewart 1996, Stewart & Krozowski 1999, Quinkler *et al.* 2001).

The $NADP^{+}$/H-dependent 11β-HSD type 1 (11β-HSD-1) enzyme functions *in vitro* as a bidirectional oxidoreductase and is expressed ubiquitously. *In vivo*, the hepatic 11β-HSD-1 acts mainly as a reductase and activates inactive cortisone to cortisol (Jamieson *et al.* 1995, 2000, Voice *et al.* 1996). 11β-HSD-1 of the guinea pig has recently been cloned and was present in all tissues

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examined, with highest levels in liver, kidney and the adrenal gland (Pu & Yang 2000).

Whereas the function of 11β-HSD-2 is widely accepted, the function of 11β-HSD-1 is not yet well understood. It is believed that 11β-HSD-1 modulates intracellular active GC concentration and occupancy of the GC receptor. In the fetal lung, the activation of cortisone to cortisol by 11β-HSD-1 is essential for the induction of surfactant synthesis (Hundertmark *et al.* 1995), in the ovary for modulation of follicular maturation during the follicular phase (Michael & Cooke 1994, Tetsuka *et al.* 1997), and in adipose tissue for differentiation of adipocytes (Tomlinson *et al.* 2001, Stewart & Tomlinson 2002). In the liver, 11β-HSD-1 plays a crucial role in GC-mediated effects such as gluconeogenesis, glycolysis and insulin sensitivity (Voice *et al.* 1996, Kotelevtsev *et al.* 1997), and it is probably involved in detoxification of nitrosamines (Maser *et al.* 1996).

Recently, we and others have suggested that the increased ratio of cortisol to cortisone in plasma and urine during adrenocorticotrophin (ACTH) infusion in man (Eisenschmid *et al.* 1987, Walker *et al.* 1992) is not caused by a direct inhibitory effect of ACTH (Diederich *et al.* 1996), but is due to an overload of 11β-HSD-2 substrates such as cortisol and corticosterone with subsequent 11β-HSD-2 inhibition (Ulick *et al.* 1992).

Stress activates the hypothalamo–pituitary–adrenal axis and the sympathetic nervous system. During stress, ACTH, corticosteroid and catecholamine concentrations are high, as are other stress hormones. The plasma corticosteroid levels are the most striking changes during stress, and are regarded as a sensitive index of stress. Corticosteroid levels are regulated by the 11β-HSD enzymes, but it is not known how the latter respond to stress situations. We have recently characterized the 11β-HSD isoenzymes in the guinea pig liver and kidney (Quinkler *et al.* 1997). In this paper we describe the effects of *in vivo* ACTH administration for 3 days on the 11β-HSDs and the *in vitro* effects of ACTH on these enzymes in guinea pig liver and kidney tissue slices.

Materials and Methods

Chemicals and solutions

Cortisol and cortisone were obtained from Sigma Chemical Co. (St Louis, MO, USA), [1,2,6,7-^{3}H(n)]-cortisol (specific activity: 70·0 Ci/mmol) from DuPont de Nemours GmbH (Bad Homburg, Germany), and [1,2(n)-^{3}H]-cortisone (specific activity: 41Ci/mmol) from Amersham International plc (Amersham, Bucks, UK). Both tritiated steroids were purified by high performance liquid chromatography (HPLC) prior to use. The steroids were dissolved in methanol and kept at −20 °C. Synacthen ($ACTH_{1–24}$) was purchased from Ciba-Geigy GmbH (Wehr, Germany), ketamine from Sanofi Ceva (Düsseldorf, Germany) and xylazine (Rompun) from Bayer AG (Leverkusen, Germany). Acetone, ethanol, n-hexane and 2-propanol (all LiChrosolv quality) were purchased from Merck GmbH (Darmstadt, Germany), methanol in LiChrosolv quality from J T Baker BV (Deventer, The Netherlands), Sep-Pak C_{18} cartridges from Waters Millipore GmbH (Eschborn, Germany) and radioactivity scintillant 'Instant Scint Gel Plus' from Packard Instruments BV (Groningen, The Netherlands).

Animals

Male Duncan–Hartley guinea pigs (weight 300 g) were provided by Moellegard Breeding Centre (Schönwalde, Germany), and kept under standard conditions and on a control diet. The study was approved by the local ethics committee and is in agreement with UK legal requirements. The animals were randomly divided into two groups. One group ($n=6$) was stimulated with 0·1 ml depot $ACTH_{1–24}$ (10 IU) by s.c. injection twice daily (at 0800 h and 1800 h) for the last 3 days before they were killed. The other group ($n=6$) received injections of 0·1 ml saline (0·9%) as a placebo at the same times. One hundred and fifty minutes after the last injection of $ACTH_{1–24}$ or saline in the morning, the animals were anaesthesized with ketamine combined with xylazine by intramuscular injection. Blood samples were obtained by heart puncture, and plasma was stored at −20 °C until analysis. The animals were killed, and the livers and kidneys quickly removed and kept in cold saline until the onset of incubation (maximum duration from death to onset of incubation was 70 min). Urine was collected over 24 h on the day before the injections started and before the animals were killed. The urine samples were stored at −20 °C until analysis. Plasma steroids were measured by HPLC or radioimmunoassay (RIA); tetrahydrocortisol (THF), 5αTHF and tetrahydrocortisone (THE) concentrations in 24-h urine samples were analyzed by RIA (Maser-Gluth *et al.* 2000). Guinea pig liver and kidney tissues were cut into 1 mm slices, and 70 mg wet tissue was weighed out for each incubation well. The incubation volume of 1 ml Krebs–Ringer–bicarbonate–glucose buffer contained 100 µl [^{3}H]-cortisol or [^{3}H]-cortisone (100 000 c.p.m. = 10^{-9} mol/l) and unlabelled cortisol or cortisone (10^{-7} mol/l). The incubation time was 90 min, and four independent incubations for each tissue and reaction direction were performed. Incubations of tissue slices were carried out in a shaking preheated (37 °C) steel chamber with a 95% O_2 and 5% CO_2 gas supply as described previously (Oelkers *et al.* 1994, Diederich *et al.* 1996).

The possibility of a direct effect of $ACTH_{1–24}$ on renal or hepatic 11β-HSD of the guinea pig was tested by measuring the conversion of cortisol to cortisone and vice versa in the presence of two concentrations of $ACTH_{1–24}$ (10^{-10} and 10^{-9} mol/l). The same amounts of tissue, radioactive tracer and unlabelled cortisol/cortisone were used as described above. For studies of enzyme kinetics we studied the renal and hepatic 11β-oxidation and

-reduction in guinea pig tissues by measuring the conversion of [^{3}H]-cortisol (100 000 c.p.m. = 10^{-9} mol/l) to [^{3}H]-cortisone and vice versa in the presence of increasing concentrations of unlabelled cortisol or cortisone (10^{-9} to 10^{-5} mol/l). For each concentration, tissue and reaction direction, three independent incubations were performed (incubation time 90 min).

Analytical procedure

The incubation was stopped by transferring the incubation set on ice and by removing the supernatant fluid. Precipitation of protein and cleaning of samples with Sep-Pak C_{18} cartridges and HPLC procedure with ^{3}H measurement were performed as described previously (Diederich *et al.* 1996). In the *in vivo* experiment steroids were extracted as mentioned above, but were spotted on a thin-layer chromatography plate that was developed in dichloromethane/methanol (15:1, v/v). The bands containing cortisol and cortisone were identified by UV light of the unlabelled carriers, cut out, transferred into scintillation vials and analyzed in a beta-counter. The interconversion of 11-hydroxy- or 11-oxosteroids by tissue slices was expressed as percentage of the total steroid measured.

Statistics

Statistical calculations were done with an SPSS program from SPSS Inc. (Chicago, IL, USA). The Duncan multiple range and the Mann–Whitney rank sum tests as well as the independent *t*-test were used.

Results

Guinea pig in vivo *experiment*

The mean weight of guinea pigs of the ACTH subgroup was 305·5 ± 11·3 g and that of the control group was 301·7 ± 15·4 g before the experiment. The animals of each group gained weight significantly during the experiment, resulting in slightly heavier ACTH-treated animals (377·5 ± 10·4 g) than control animals (370·5 ± 20·5 g). There was no significant difference in weight between the ACTH-treated animals and the control animals.

Serum cortisol concentrations rose significantly from 525 ± 194 nmol/l to 2679 ± 640 nmol/l following ACTH treatment for 3 days. Plasma cortisone concentrations rose likewise from 55 ± 18 nmol/l (control guinea pigs) to 336 ± 62 nmol/l in ACTH-treated animals (Table 1). The serum cortisol/cortisone ratio was not altered significantly in ACTH-treated or control animals. Other steroids measured (progesterone, 17α-hydroxyprogesterone and androstenedione) increased significantly following *in vivo* ACTH treatment (Table 1). Plasma dehydroepiandrosterone sulphate (DHEA-S) concentrations were below the assay detection limit in control animals and slightly above the limit in ACTH-treated animals.

Table 1 Serum concentrations of different steroids in ACTH-treated and control guinea pigs. Values are means ± S.D. (n=6 in each group)

	Control animals	ACTH-treated animals	Control vs ACTH (*t*-test)
Serum concentration (nmol/l)			
Cortisol	525 ± 194	2679 ± 640	P<0·001
Cortisone	55 ± 18	336 ± 62	P<0·001
Progesterone	2·7 ± 0·3	10·9 ± 2·1	P<0·001
17α-Hydroxyprogesterone	0·35 ± 0·15	2·32 ± 0·6	P<0·001
Androstenedione	2·3 ± 0·6	10·7 ± 4·7	P<0·005
DHEA-S	<0·135	0·189 ± 0·014	P<0·001

The urinary excretion of cortisol, cortisone, THF, 5αTHF and THE increased significantly under ACTH treatment (Table 2). Surprisingly the urinary cortisol/cortisone ratio did not increase after ACTH treatment, whereas the urinary excretion of THF+5αTHF/THE showed a significant increase compared with the ratio before ACTH treatment and to the control group (Fig. 1). The urinary ratio 5αTHF/THF, representing the balance between 5α-reductases and 5β-reductase activities, remained unchanged in control and ACTH-treated animals before and after treatment (Table 2).

Guinea pig in vitro *experiments*

Incubations with [^{3}H]-cortisol to [^{3}H]-cortisone and vice versa in the presence of increasing concentrations of unlabelled cortisol or cortisone (10^{-9} to 10^{-5} mol/l) in untreated guinea pig liver and kidney tissue slices showed typical enzyme kinetics with no substrate overload (data not shown).

Three days of *in vivo* ACTH treatment significantly increased the *in vitro* 11β-reductase activity in guinea pig liver (50·2% to 70·3%) and kidney (39·6% to 56·8%) slices, whereas the 11β-oxidative activity was significantly decreased in both liver (33·7% to 23·9%) and kidney (33·9% to 28·6%) (Fig. 2). Due to the high conversion of substrate, and consequent difficulties in accurately measuring absolute conversion rates, the results observed may underestimate the difference between control and treatment groups

In vitro incubations of liver and kidney slices with increasing concentrations of $ACTH_{1–24}$ had no significant effect on the interconversion of cortisol to cortisone (Table 3).

Discussion

The pituitary–adrenal system of the guinea pig (*Cavia porcellus*) is different from other rodents in several respects (Keightley & Fuller 1996). The total plasma cortisol concentration in unstressed animals ranges between 260

Table 2 Daily urinary excretion of free cortisol, cortisone, THF, 5αTHF and THE in ACTH-treated and control guinea pigs. Values are means ± S.D. (n=6 in each group). Paired and unpaired t-tests were used

		Control group (NaCl) (μg/24 h)	ACTH-treated group (μg/24 h)	Control vs ACTH (t-test)
Urinary steroid profiles				
Cortisol	Before injection	44·5 ± 13·6	51·4 ± 17·9	P=0·468
	3rd day of injection (ACTH or NaCl)	30·1 ± 8·2	364·3 ± 88·5	P<0·001
	t-test (before vs after injections)	P=0·051	P<0·001	
Cortisone	Before injection	1·7 ± 0·4	2·5 ± 0·8	P=0·065
	3rd day of injection (ACTH or NaCl)	1·2 ± 0·8	22·1 ± 10·7	P<0·001
	t-test (before vs after injections)	P=0·143	P<0·001	
Cortisol/cortisone ratio	Before injection	25·8 ± 7·1	20·7 ± 2·3	P=0·125
	3rd day of injection (ACTH or NaCl)	35·9 ± 22·4	20·4 ± 10·2	P=0·168
	t-test (before vs after injections)	P=0·194	P=0·943	
THF	Before injection	8·8 ± 2·7	8·9 ± 3·3	P=0·940
	3rd day of injection (ACTH or NaCl)	7·0 ± 1·7	41·2 ± 11·0	P=0·001
	t-test (before vs after injections)	P=0·272	P=0·001	
5αTHF	Before injection	4·6 ± 0·8	4·9 ± 0·8	P=0·506
	3rd day of injection (ACTH or NaCl)	3·6 ± 0·6	16·7 ± 3·2	P<0·001
	t-test (before vs after injections)	P=0·073	P<0·001	
THE	Before injection	7·0 ± 1·5	8·2 ± 2·7	P=0·385
	3rd day of injection (ACTH or NaCl)	6·4 ± 2·1	24·8 ± 7·0	P=0·001
	t-test (before vs after injections)	P=0·605	P<0·001	
5αTHF/THF	Before injection	0·55 ± 0·13	0·65 ± 0·4	P=0·572
	3rd day of injection (ACTH or NaCl)	0·53 ± 0·11	0·42 ± 0·08	P=0·058
	t-test (before vs after injections)	P=0·812	P=0·222	
(THF+5αTHF)/ THE	Before injection	1·90 ± 0·28	1·82 ± 0·54	P=0·471
	3rd day of injection (ACTH or NaCl)	1·72 ± 0·26	2·39 ± 0·33	P=0·003
	t-test (before vs after injections)	P=0·330	P<0·045	

and 690 nmol/l (Fujieda *et al.* 1982, Fenske 1997), and free cortisol concentrations are at least three times higher than in humans (Fujieda *et al.* 1982). One reason for the high circulating cortisol concentration may be the low affinity of the guinea pig GC receptor to cortisol (Hodgson & Funder 1978, Kraft *et al.* 1979). In spite of high cortisol levels, the plasma ACTH concentration is comparable with that in other species (Martin *et al.* 1980), but the biological potency of guinea pig ACTH is increased (Keightley *et al.* 1991). The guinea pig MC receptor shows no differences from that of other species (Myles & Funder 1994). We found that the guinea pig possesses a very strong 11β-HSD-2 activity in the kidney (Quinkler *et al.* 1997), which could be the major protecting mechanism for the MC receptor.

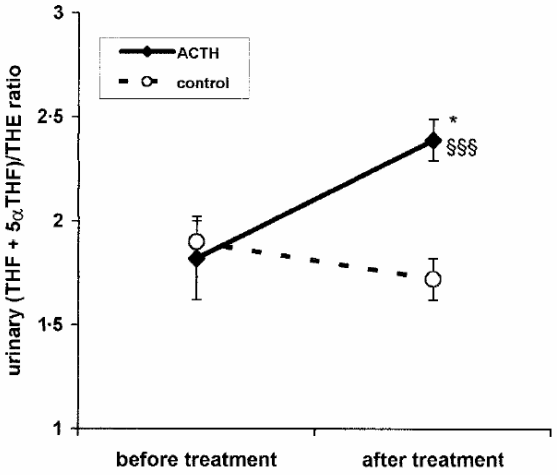

Figure 1 Urinary (THF+5αTHF)/THE ratio of six ACTH- and NaCl-treated guinea pigs. Values are means ± S.E.M. Significant differences (§§§P<0·005) of the ratios after treatment in ACTH-treated versus the control group. Significant increase (*P<0·05) of the ratio in the ACTH-treated group before versus after the injections.

It is not known how 11β-HSD activity and its reaction directions are influenced in chronic stress situations. Until now, only a few studies have addressed this topic. Nwe *et al.* (2000) investigated the 11β-HSD-1 oxidative activity of testis and liver during stress in rats. They did not investigate reductase activity, which is the main function of 11β-HSD-1, and they used only homogenates. Tissue slices are preferrable to homogenates, because homogenization of tissue changes 11β-HSD-1 activity from reduction to oxidation (Oelkers *et al.* 1994, Bach *et al.* 1996). We therefore investigated the effect of 3 days of *in vivo* ACTH treatment on the interconversion of cortisol and cortisone in liver and kidney slices of the guinea pig.

Three days of ACTH treatment increased adrenal steroidogenesis. Besides increased cortisol and cortisone

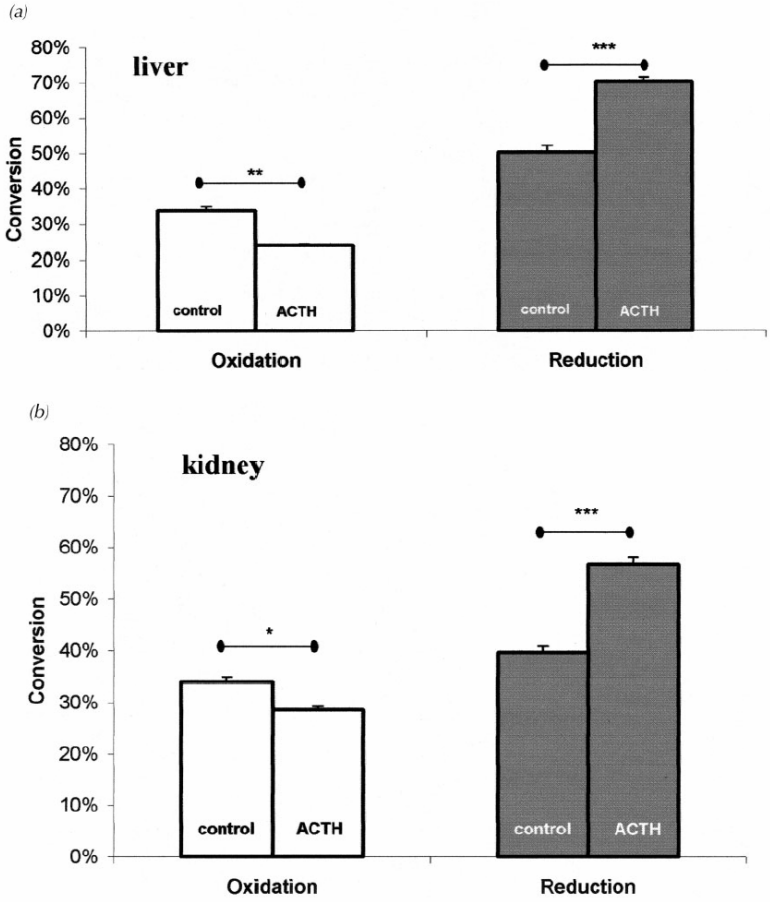

Figure 2 Conversion (%) of [^{3}H]-cortisol and [^{3}H]-cortisone in guinea pig (*a*) liver tissue slices (n=6) and (*b*) kidney tissue slices (n=6) of sham- and ACTH-treated animals; 100 000 c.p.m. [^{3}H]-cortisol or [^{3}H]-cortisone and 10^{-7} mol/l unlabelled cortisol or cortisone were used. The incubation time was 90 min. Values are means ± S.E.M. (a) $^{**}P<0{\cdot}005$, $^{***}P<0{\cdot}001$; (b) $^{*}P<0{\cdot}05$, $^{***}P<0{\cdot}001$.

plasma concentrations (Table 1), we observed an increased production of progesterone, 17α-hydroxyprogesterone and androstenedione suggesting an activated 17α-hydroxylase and 17,20-lyase activity of P450c17 enzyme by ACTH treatment (Provencher *et al.* 1992). The DHEA-S plasma concentration was undetectable in untreated animals and was just measureable in ACTH-treated animals. The P450c17 enzyme of the guinea pig seems to prefer the progesterone–17α-hydroxyprogesterone–androstenedione pathway similar to rats (Shinzawa *et al.* 1985). This is in contrast to the human P450c17, which favours the pregnenolone–17α-hydroxypregnenolone–DHEA pathway.

In liver and kidney tissue slices, 11β-HSD dehydrogenase activity decreased after *in vivo* ACTH treatment, whereas the 11β-HSD reductase activity increased considerably compared with the control group. The equilibrium of the reaction was shifted towards the active 11-hydroxy side (Fig. 2). In guinea pig liver, 11β-HSD1 mRNA expression and enzyme activity is predominant (Quinkler *et al.* 1997, Pu & Yang 2000). Therefore, the increase of the reduction of cortisone is most likely due to an increase in 11β-HSD-1 activity conferred by GCs (Jamieson *et al.* 1995, Liu *et al.* 1996). The preference for reduction could be caused by an increase of reduced cosubstrate (NADPH) and a lower intracellular pH, due to anaerobic glycolysis in stress situations. At lower pH (6·0) the reductase reaction is favoured over the dehydrogenase reaction (pH 8·0) (Brown *et al.* 1993). A direct transcriptional regulation could also be a possibility, but would not explain the reduced oxidation and increased reductive activity of one enzyme. Therefore, this change in enzyme

Table 3 Percentage conversion of cortisol (F) to cortisone (E) and vice versa in kidney and liver tissue slices with increasing concentrations of $ACTH_{1-24}$ *in vitro*. Values are means ± S.E.M. (n=3). Percentage conversion of controls was set at 100%

		$ACTH_{1-24}$	
	Control (%)	10^{-10} mol/l	10^{-9} mol/l
Kidney			
Oxidation (F to E)	100 ± 7·8	96·9 ± 8·8	87·1 ± 3·6
Reduction (E to F)	100 ± 13·9	83·6 ± 9·6	91·7 ± 1·7
Liver			
Oxidation (F to E)	100 ± 3·7	112 ± 13·5	104·6 ± 6·6
Reduction (E to F)	100 ± 11·1	117 ± 6·9	106·4 ± 10·4

activity seems to correlate better with a shift of the equilibrium to the reductive side than with direct transcriptional changes.

We also observed marked oxidative activity in liver tissue slices. This can be explained by the activity of a third isoenzyme in guinea pig liver acting as a dehydrogenase (Quinkler *et al.* 1997). Since the oxidative activity was significantly decreased after *in vivo* ACTH treatment, an inhibition of this third 11β-HSD isoenzyme by ACTH treatment seems possible. This 11β-HSD isoenzyme could constitute a compensatory metabolic pathway for the poor cortisol metabolism by other enzymatic systems (e.g. ring A reduction) in the guinea pig liver (Abel *et al.* 1993).

Besides 11β-HSD-2, the guinea pig kidney expresses 11β-HSD-1 similar to rat and mouse kidney (Quinkler *et al.* 1997, Pu & Yang 2000). *In vivo* ACTH treatment increases renal reductive activity, possibly due to an activated 11β-HSD-1 activity. Although it is known that in the rat kidney 11β-HSD-2 is induced by GCs (Li *et al.* 1996), it seems as if the activation of type 1 isoenzyme would predominate in the guinea pig. It is also possible that the type 2 isoenzyme is inhibited by ACTH-induced steroids, e.g. progesterone and its metabolites, or by corticosterone (Diederich *et al.* 1996, Quinkler *et al.* 1999). Plasma cortisone concentration rises to over 300 nmol/l after ACTH treatment (Table 1) and cortisone has a lower corticosteroid-binding globulin affinity than cortisol. Therefore, end-product inhibition of 11β-HSD-2 by cortisone, which was described previously (Rusvai & Náray-Fejes-Tóth 1993, Albiston *et al.* 1994, Stewart *et al.* 1995), may also be an important reason for the decrease of renal 11β-HSD-2 activity *in vivo*. The physiological role of a decreased 11β-HSD-2 activity in response to stress is not clear. On the one hand, the body would need higher levels of oxidase activity in cells expressing the MC receptor to cope with increased cortisol levels and protect the MC receptor. But, on the other hand, even these cells may need a higher intracellular level of active GCs to respond adequately to stress. In addition, fluid retention is critical in stress, as we see it clinically in shock situations like septic or haemorrhagic shock, and traumata. Therefore an increased activation of the MC receptor would make sense.

A study in 11β-HSD-1 knock-out mice showed the importance of the 11β-HSD-1 isoenzyme for GC action: a lack of this enzyme leads to a relative intracellular GC deficiency (Kotelevtsev *et al.* 1997). Since ACTH had no effect on the bidirectional enzyme activities when added *in vitro* to liver or kidney slices (Table 3), the activation of the 11β-HSD-1 after *in vivo* ACTH application is most likely mediated by cortisol itself or by other ACTH-induced steroids (Hammami & Siiteri 1991, Walker *et al.* 1994, Jamieson *et al.* 1995, Voice *et al.* 1996). This is in accordance with the finding that 11β-HSD-1 activity is increased by dexamethasone in a rat hepatoma cell line (2S FAZA) (Voice *et al.* 1996), in primary culture of rat hepatocytes (Liu *et al.* 1996) and in rat liver *in vivo* (Jamieson *et al.* 1999). It was recently demonstrated that GC treatment caused a time- and dose-dependent increase in 11β-HSD-1 mRNA and activity in primary cultures of human osteoblasts (Cooper *et al.* 2002).

Nevertheless, this activation of 11β-HSD-1 in stress situations could be an important regulatory principle in many organ systems. This enhanced 'GC tonus' is essential for stress adaptation, e.g. GC-induced increase of gluconeogenesis in the liver. Due to the increased conversion of inactive cortisone to active cortisol, the liver seems to be an important cortisol production site in stress situations, besides the adrenals.

This observation was underlined by the significantly elevated urinary THF+5αTHF/THE ratio in the ACTH-treated group of guinea pig as a marker of 11β-HSD-1 activity (Fig. 2a). It has recently been proposed that the urinary ratio of THF+5αTHF/THE may also be an accurate marker for renal 11β-HSD-2 in humans (Ferrari *et al.* 2001). Due to different isoenzyme expression in the guinea pig kidney (11β-HSD-1 in addition to 11β-HSD-2) (Quinkler *et al.*1997, Pu & Yang 2000), this cannot be easily transferred to the guinea pig. We rather interpret this ratio as a marker for hepatic 11β-HSD-1 activity (Palermo *et al.* 1996, Quinkler *et al.* 2000). The urinary 5αTHF/THF ratio, representing the balance between 5α-reductases and 5β-reductase activities, did not change under ACTH treatment (Table 2). This implies that the increased THF+5αTHF/THE ratio was not influenced by 5α-reductases or 5β-reductase activities but represents an increased 11β-HSD-1 reductase activity. Surprisingly, the total amount of urinary tetrahydrometabolites was rather small compared with the human in relation to the cortisol concentration. This can be explained by the relatively poor cortisol metabolism to ring A-reduced metabolites in the guinea pig liver (Abel *et al.* 1993). Therefore these ratios need further assessment in this species.

The urinary cortisol/cortisone ratio in the guinea pig is high (approximately 20), whereas in humans it is low (approximately 0·5). The urinary ratio cortisol/cortisone is an excellent marker for 11β-HSD-2 in humans, because the human kidney expresses only 11β-HSD-2. But the ratio is a poor index for 11β-HSD-2 activity in the guinea pig, because both 11β-HSD enzymes are expressed in the kidney and contribute to the urinary cortisol/cortisone ratio. Therefore, it is difficult to compare human and guinea pig urinary cortisol/cortisone ratios. In situations of unchanged urinary cortisol/cortisone ratio in ACTH-treated animals, the increase in THF+5αTHF/THE ratio reflects an increase in 11β-HSD-1 activity in the liver.

In summary, we have shown that *in vivo* ACTH treatment for 3 days increases the hepatic and renal 11β-reductase activity in the guinea pig. This activation is probably due to increased concentrations of cortisol or of other ACTH-dependent GCs rather than to ACTH itself. The stress-induced activation of the hormonally inactive cortisone to active cortisol in liver, kidney and other 11β-HSD-1-expressing organs seems to be an important local tissue regulatory mechanism besides the adrenal cortisol *de novo* synthesis. Up to now, this mechanism, which could also be present in other species such as the human, has not been noted to be involved in the elevation of cortisol by ACTH in humans.

Acknowledgements

This work was supported partly by grant DI 741/1-3 from the Deutsche Forschungsgemeinschaft (DFG). M Q is supported by a postdoctoral fellowship (QU142/1-1) of the DFG.

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Received 10 October 2002
Accepted 23 October 2002

3.1.3 Inhibitoren der 11β-HSD

3. Diederich S, Grossmann C, Hanke B, **Quinkler M**, Herrmann M, Bähr V, Oelkers W. In the search for specific inhibitors of human 11β-hydroxysteroid-dehydrogenases (11β-HSDs): Chenodeoxycolic acid selectively inhibits 11β-HSD1. *Eur.J.Endocrinol.* **2000**, 142: 200-207.

European Journal of Endocrinology (2000) **142** 200–207 ISSN 0804-4643

EXPERIMENTAL STUDY

In the search for specific inhibitors of human 11β-hydroxysteroid-dehydrogenases (11β-HSDs): chenodeoxycholic acid selectively inhibits 11β-HSD-I

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Abstract

Objective: Selective inhibitors of 11β-hydroxysteroid-dehydrogenase type I may be of therapeutical interest for two reasons: i) 9α-Fluorinated 11-dehydrosteroids like 11-dehydro-dexamethasone (DH-D) are rapidly activated by human kidney 11β-hydroxysteroid-dehydrogenase type II (11β-HSD-II) to dexamethasone (D). If the same reaction by hepatic 11β-HSD-I could be selectively inhibited, DH-D could be used for selective renal immunosuppressive therapy. ii) Reduction of cortisone to cortisol in the liver may increase insulin resistance in type 2 diabetes mellitus, and inhibition of the enzyme may lead to a decrease in gluconeogenesis.

Therefore, we characterized the metabolism of DH-D by human hepatic 11β-HSD-I and tried to find a selective inhibitor of this isoenzyme.

Methods: For kinetic analysis of 11β-HSD-I, we used microsomes prepared from unaffected parts of liver segments, resected because of hepatocarcinoma or metastatic disease. For inhibition experiments, we also tested 11β-HSD-II activity with human kidney cortex microsomes. The inhibitory potency of several compounds was evaluated for oxidation and reduction in concentrations from 10^{-9} to 10^{-5} mol/l.

Results: Whereas D was not oxidized by human liver microsomes at all, cortisol was oxidized to cortisone with a maximum velocity (V_{max}) of 95 pmol/mg per min. The reduction of DH-D to D (V_{max} = 742 pmol/mg per min, Michaelis–Menten constant (K_m) = 1.6 μmol/l) was faster than that of cortisone to cortisol (V_{max}=187 pmol/mg per min). All reactions tested in liver microsomes showed the characteristics of 11β-HSD-I: K_m values in the micromolar range, preferred cosubstrate NADP(H), no product inhibition. Of the substances tested for inhibition of 11β-HSD-I and -II, chenodeoxycholic acid was the only one that selectively inhibited 11β-HSD-I (IC_{50} for reduction: 2.8×10^{-6} mol/l, IC_{50} for oxidation: 4.4×10^{-6} mol/l), whereas ketoconazole preferentially inhibited oxidation and reduction reactions catalyzed by 11β-HSD-II. Metyrapone, which is reduced to metyrapol by hepatic 11β-HSD-I, inhibited steroid reductase activity of 11β-HSD-I and -II and oxidative activity of 11β-HSD-II. These findings can be explained by substrate competition for reductase reactions and by product inhibition of the oxidation, which is a well-known characteristic of 11β-HSD-II.

Conclusions: Our *in vitro* results may offer a new concept for renal glucocorticoid targeting. Oral administration of small amounts of DH-D (low substrate affinity for 11β-HSD-I) in combination with chenodeoxycholic acid (selective inhibition of 11β-HSD-I) may prevent hepatic first pass reduction of DH-D, thus allowing selective activation of DH-D to D by the high affinity 11β-HSD-II in the kidney. Moreover, selective inhibitors of the hepatic 11β-HSD-I, like chenodeoxycholic acid, may become useful in the therapy of patients with hepatic insulin resistance including diabetes mellitus type II, because cortisol enhances gluconeogenesis.

European Journal of Endocrinology **142** 200–207

Introduction

One of the goals of a rational therapy is to increase drug specificity by directing drug action toward a desired target organ or tissue with the exclusion of other sites of action. Such selectivity can be expected to minimize side effects and prevent toxicity. One approach is the chemical modification of an active drug to an inactive precursor or prodrug, which should then be converted into the active compound by an enzyme specifically present in the target organ.

11β-Hydroxysteroid dehydrogenases (11β-HSD) are microsomal enzymes catalyzing the conversion of active glucocorticoids (GCs) to their receptor inactive

11-dehydro products and vice versa (1, 2). Two isoenzymes of 11β-HSD have been characterized and cloned in human tissues (3, 4). Since enzyme kinetics, sites of expression and molecular biology are markedly different between 11β-HSD-I and -II (5), some authors disapprove of the term 'isoenzymes'.

11β-HSD-II is mainly found in mineralocorticoid (MC) target tissues (kidney, colon, salivary glands) and the placenta. Recently, expression of 11β-HSD-II has also been demonstrated in discrete areas of 'non-classical' MC target organs (e.g. sodium-transporting epithelia of the lung, 6). As reverse-transcriptase polymerase chain reaction (RT-PCR) of RNA from the whole tissue of those organs has not shown any amplification (4), expression of 11β-HSD-II in 'non-classical' MC target organs seems to be very low and is probably unimportant for the total glucocorticoid metabolism of these organs. 11β-HSD-II mainly oxidizes (inactivates) physiological GCs (cortisol (F) in humans, corticosterone (B) in rat), uses NAD as a cosubstrate and has a K_m-value in the nanomolar range that is close to physiologically relevant concentrations of free F or B. 11β-HSD-II has a well-characterized role in the kidney, where it provides MC selectivity to aldosterone, which binds with the same affinity as F to the MC receptor (7), but is not an 11β-HSD-II substrate. We have previously shown that 9α-fluorinated steroids (dexamethasone and 9α-fluoro-F) are also oxidized by human kidney 11β-HSD-II, but the main reaction for these steroids is the reduction of the 11-keto group (8–10). These results disproved the dogma of a unidirectional (oxidative) 11β-HSD-II function and were later confirmed in experiments with cells selectively transfected with 11β-HSD-II (11). As 11-dehydrosteroids like cortisone, prednisone or 11-dehydro-dexamethasone (DH-D) do not bind to the GC receptor (12), DH-D or other 9α-fluorinated 11-dehydrosteroids might be interesting GC prodrugs being selectively activated in organs expressing 11β-HSD-II, especially kidney and colon. However, targeting 11-dehydrosteroids to the kidney would only work if the liver did not convert these prodrugs into active glucocorticoids by 11β-HSD-I.

11β-HSD-I is found in many tissues, with the highest expression in the liver (13). It seems to be colocalized with the GC receptor (14). In typical MC target tissues, its expression is low (kidney medulla) or undetectable (kidney cortex, salivary gland, sigmoid and rectal colon) (13). It has a lower affinity for physiological steroids (K_m in the micromolar range) than the type II isoenzyme and prefers NADP(H) as its cosubstrate. In tissue homogenates and microsomal fractions, 11β-HSD-I works bidirectionally, oxidizing and reducing physiological GCs (15). In contrast, in intact dividing cells expressing 11β-HSD-I (16–18) and in primary cultures of hepatocytes (19), the enzyme seems to act predominantly as an 11β-reductase regenerating active GCs from their 11-keto metabolites.

Although human 11β-HSD-I was cloned some years before the type II isoenzyme, exact kinetic data are scant for the type I enzyme. Most of the studies cited above were done with rat 11β-HSD-I (microsomes or homogenates, transfected cells, hepatocyte cultures). As yet, human 11β-HSD-I was only examined in transfected cells. Results obtained in such cell preparations can be different from those in tissue homogenates or microsomes. Selective inhibitors of 11β-HSD-I may have a role in the therapy of patients with hepatic insulin resistance (20–22) or with central obesity (23) and could be a promising combination with 9α-fluorinated dehydrosteroids like dehydro-dexamethasone in the scope of renal glucocorticoid targeting (24).

For the present study we prepared human hepatic microsomes in order to analyze enzyme kinetics of human 11β-HSD-I and to find a selective inhibitor of this enzyme.

Materials and methods

Materials

The following steroids and substances were purchased from Sigma Chemical Co. (St Louis, MO, USA): cortisol (F), cortisone (E), dexamethasone (D), chenodeoxycholic acid (CDCA), lithocholic acid, 18β-glycyrrhetinic acid, carbenoxolone, 11α-OH-progesterone (4-pregnen-11α-ol-3,20-dione), 11β-OH-progesterone (4-pregnen-11β-ol-3,20-dione), 5α-dihydro-progesterone (5α-pregnan-3,20-dione), 5β-dihydro-progesterone (5β-pregnan-3,20-dione), 20β-OH-progesterone (4-pregnen-20β-ol-3-one), deoxycorticosterone (DOC, 4-pregnen-21-ol-3,20-dione), the cofactors NAD/NADH and NADP/NADPH, D-glucose-6-phosphate (monosodium salt) and sucrose. 3α,5β-TH-DOC (5β-pregnan-3α,21-diol-20-one) and 3α,5β-TH-progesterone (5β-pregnan-3α,21-diol-20-one) were obtained from Steraloids Inc. (Wilton, NH, USA), metyrapone from Fluka AG (Buchs, Switzerland) and ketoconazole from Biotrend GmbH (Cologne, Germany). Unlabelled 11-dehydro-dexamethasone (DH-D) was a gift from Schering AG (Berlin, Germany). [1,2,6,7-^{3}H]-F (specific activity: 70.0 Ci/mmol) was obtained from DuPont de Nemours GmbH (Bad Homburg, Germany). [1,2-^{3}H]-E (specific activity: 41 Ci/mmol) and [1,2,4,6,7-^{3}H]-D (specific activity: 70 Ci/mmol) were purchased from Amersham International plc (Bucks, UK). [1,2,4,6,7-^{3}H]-DH-D was synthesized in our laboratory from tritiated D by oxidation with chromium-6-oxide as described previously (10). All tritiated steroids were purified by HPLC prior to use.

Glucose 6-phosphate dehydrogenase (from Leuconostoc mesenteroides) was purchased from Boehringer (Mannheim, Germany); sodium hydrogenphosphate dihydrate and sodium hydrogenphosphate monohydrate were obtained from Merck Ltd (Darmstadt, Germany).

Thin layer chromatography (TLC) plates coated with silicagel 60 (F_{254}) were obtained from Merck

(Darmstadt, Germany); dichloromethane–methanol (75 + 5; Lichrosolv, Merck), was used as the mobile phase. The scintilat, Instagel Plus, was purchased from Packard (Frankfurt, Germany).

Preparation of microsomes

Human liver tissue was taken from unaffected liver segments removed during surgical resection in the treatment of hepatocarcinoma or metastatic disease. Microsomes were prepared from liver tissue of 15 different patients (9 male and 6 female patients between 25 and 82 years old; total amount of tissue, 80 g). The whole tissue was homogenized in 0.01 mol/l sodium phosphate buffer, containing 0.25 mol/l sucrose. All subsequent steps were performed at 0–4 °C. Microsomes were prepared by differential centrifugation as described previously for kidney microsomes (10). The microsomes were used within 6 months, and no loss in enzyme activity was seen up to this time. Since we used the same microsome pool for all reactions, the kinetic parameters are comparable.

Enzyme kinetics of 11β-HSD-I

Enzyme kinetics of 11β-HSD-I for the steroid pairs F/E and D/DH-D were determined by the method described for human kidney microsomes (10). In brief, the cosubstrate concentration was always 10^{-3} mol/l, pH was 8.5 for all dehydrogenase reactions and 6.0 for all oxo-reductase reactions. The amount of labeled steroids was constant in every experiment (100 000 c.p.m.). The incubation was started by addition of microsomes to 10 min preincubated wells containing all the assay components except the enzyme preparation. Incubations were done at 37 °C in a shaking water bath and terminated by addition of 2 ml cold methanol and by rapid transfer of the multiwell incubation plate on ice. Each experiment was done at least in triplicate. By varying the amount of enzyme preparation and the reaction time, initial velocities of each reaction were always in the linear range. D was not oxidized to DH-D, although we used high protein concentrations and a long incubation time. For kinetic analyses we added increasing amounts of the corresponding unlabeled steroid. For the reduction of E to F, no linear time kinetics could be obtained by changing the protein concentration or the incubation time. Using an NADH/NADPH-regenerating system (10^{-2} mol/l glucose 6-phosphate and 10 units glucose 6-phosphate dehydrogenase for each incubation), we could perform clearcut kinetic analyses for this reaction.

Analytical procedure

The first analytical procedure for each reaction was performed using HPLC (25). We were unable to detect any other metabolites than the 11-oxo- or 11-hydroxysteroids after incubation.

For the numerous measurements of enzyme kinetics and inhibition experiments, we used the TLC method developed by our group (10): steroids were extracted with Sep Pak cartridges and dissolved in 50 μl methanol containing a mixture of the corresponding unlabeled steroids (F and E or D and DH-D). This solution was applied to TLC plates and developed with dichloromethane–methanol as the mobile phase. The spots were identified under UV light, cut out and transferred to scintillation vials containing 1 ml H_2O and 15 ml Instagel Plus. Measurement of percentage conversion rates allowed the determination of the initial velocity (pmol/mg per min). We chose the Hanes–Woolf transformation for the calculations of K_m and V_{max} values (10).

Inhibition experiments (11β-HSD-I and 11β-HSD-II)

Different substances (Table 1) were tested for their inhibiting potency on 11β-oxidation and 11β-reduction of 11β-HSD-I (human liver microsomes) and 11β-HSD-II (human kidney cortex microsomes). Previous studies of our group (10) and molecular biological examinations (13) have proven that human kidney cortex microsomes exclusively possess 11β-HSD-II activity.

For each reaction, incubation conditions were varied in such a way that initial velocities in the linear range could be measured and conversion rates in the absence of inhibitors were as high as possible. The different reactions were tested with the preferred substrates and cosubstrates, and substrate concentrations were chosen in the range of the specific K_m values. The incubation conditions were as follows. Oxidation by 11β-HSD-I: substrate, 0.98 μmol/l F; cosubstrate, 1 mmol/l NADP; protein concentration, 0.15 mg/ml; incubation time, 50 min. Reduction by 11β-HSD-I: substrate, 1.6 μmol/l DH-D; cosubstrate, 1 mmol/l NADPH; protein concentration, 0.03 mg/ml; incubation time, 30 min. Oxidation by 11β-HSD-II: substrate: 25 nmol/l F; cosubstrate, 1 mmol/l NAD; protein concentration, 0.03 mg/ml; incubation time, 30 min. Reduction by 11β-HSD-II: substrate, 65 nmol/l DH-D; cosubstrate, 1 mmol/l NADH; protein concentration, 0.0075 mg/ml, incubation time, 30 min.

Each inhibiting substance was tested in concentrations from 10^{-9} to 10^{-5} mol/l. For metyrapone, we also tested the inhibiting potency up to 10^{-3} mol/l. The percentage inhibition of the reaction was calculated by measuring the decrease in product formation in the presence of the inhibitor compared with product formation in the controls. Dose–response curves, showing percentage inhibition versus log concentration of inhibitor, were plotted. From these curves, the concentrations that caused 50% inhibition of the reaction rate were determined and reported as IC_{50} values.

Statistical calculations were done with an SPSS program from SPSS Inc. (Chicago, IL, USA); the Duncan multiple range test was used.

Table 1 Inhibitors of human 11β-HSD-I (liver microsomes) and 11β-HSD-II (kidney cortex microsomes). Substrate for oxidative reactions of both isoenzymes: cortisol (F); substrate for reductase reactions: 11-dehydro-dexamethasone (DH-D). IC_{50} values (mol/l) are presented. All measurements were in the linear part of individual reactions, representing initial velocities (V_o). Three independent measurements of V_o were done for each concentration of inhibitor tested. All substances were tested in concentrations from 10^{-9} to 10^{-5} mol/l except metyrapone which was tested from 10^{-9} to 10^{-3} mol/l.

	11β-HSD-I		11β-HSD-II	
Inhibitor	F	DH-D	F	DH-D
Glycyrrhetinic acid	4.0×10^{-8}	3.1×10^{-8}	8.0×10^{-9}	1.2×10^{-8}
Carbenoxolene	6.0×10^{-8}	3.0×10^{-8}	8.4×10^{-9}	5.9×10^{-9}
11α-OH-progesterone	2.8×10^{-7}	5.1×10^{-7}	2.1×10^{-9}	1.1×10^{-8}
11β-OH-progesterone	4.4×10^{-7}	1.3×10^{-6}	6.8×10^{-9}	1.5×10^{-8}
Progesterone	1.7×10^{-6}	8.4×10^{-6}	4.8×10^{-8}	4.6×10^{-8}
5α-Dihydro-progesterone	3.8×10^{-6}	6.5×10^{-6}	2.4×10^{-7}	4.5×10^{-7}
5β-Dihydro-progesterone	1.6×10^{-6}	3.4×10^{-6}	1.1×10^{-6}	3.0×10^{-6}
20β-OH-progesterone	1.9×10^{-6}	6.1×10^{-6}	5.8×10^{-6}	4.2×10^{-7}
3α,5β-TH-progesterone	2.6×10^{-6}	4.4×10^{-6}	no inhibition	8.0×10^{-6}
Deoxycorticosterone (DOC)	4.4×10^{-6}	4.0×10^{-6}	9.9×10^{-8}	1.9×10^{-8}
3α,5β-TH-DOC	2.8×10^{-6}	2.2×10^{-6}	$>10^{-5}$	7.1×10^{-6}
Lithocholic acid	2.7×10^{-6}	2.4×10^{-6}	$>10^{-5}$	$>10^{-5}$
Chenodeoxycholic acid	4.4×10^{-6}	2.8×10^{-6}	no inhibition	no inhibition
Metyrapone	no inhibition	3.1×10^{-3}	$>10^{-3}$	6.1×10^{-3}
Ketoconazole	no inhibition	$>10^{-5}$	9.8×10^{-6}	2.1×10^{-6}

No inhibition, no significant effect on reaction velocity at the highest concentration tested. $>10^{-5}$ (or $>10^{-3}$), significant inhibition at the highest concentration tested but it was too low to determine IC_{50}.

Results

All 11β-HSD enzymatic reactions in human liver microsomes prefer NADP or NADPH as cosubstrates (Table 2). The reduction of 11-dehydro-dexamethasone (DH-D) to dexamethasone (D) was clearly NADPH dependent, even if we chose a substrate concentration near the K_m value of 11β-HSD-II (0.15 μmol/l, Fig. 1).

The percentage conversion of 0.15 μmol/l DH-D was not diminished by co-incubation with 3 μmol/l cortisol or dexamethasone, and 11β-oxidation of 0.15 μmol/l cortisol was not diminished by 3 μmol/l cortisone or 11-dehydro-dexamethasone (data not shown). These results demonstrate that oxidation and reduction of substrates by 11β-HSD-I is not inhibited by a 20-fold excess of the corresponding reaction products. In contrast, renal 11β-HSD-II is characterized by 'end-product inhibition' (10). Although we optimized the conditions for the oxidation of dexamethasone (low substrate concentration, high protein concentration, long incubation time), we did not find any oxidation of dexamethasone to 11-dehydro-dexamethasone by liver microsomes (Table 2). Comparing the V_{max} values, the 11-keto reduction of 11-dehydro-dexamethasone by 11β-HSD-I is much more effective than that of cortisone (bear in mind that we did not add the NADH/NADPH regenerating system for the reduction of 11-dehydro-dexamethasone to dexamethasone). Thus, with dexamethasone and 11-dehydro-dexamethasone as substrates, the hepatic 11β-HSD-I is a pure 11-keto-reductase. For the steroid pair cortisol/cortisone, reduction is also the preferred reaction (see the

Table 2 Kinetic data of reactions tested in human liver microsomes: 11β-oxidation of cortisol (F) to cortisone (E) and of dexamethasone (D) to 11-dehydro-dexamethasone (DH-D), and vice versa. Oxidation of D was unmeasurably low even under optimized conditions. *With NADH/NADPH regenerating system. Values are means ± S.D.

	F ⇌ E		D ⇌ DH-D	
Reaction	Oxidation	Reduction*	Oxidation	Reduction
Cosubstrate	NADP	NADPH	–	NADPH
K_m (μmol/l)	0.98 ± 0.14	1.4 ± 0.08	–	1.6 ± 0.1
V_{max} (pmol/mg/min)	94.5 ± 4.4	187 ± 4.3	–	741 ± 24
$V_{max,ox}/V_{max,red}$	0.51		–	

The correct unit for the maximum velocity (V_{max}) is pmol/mg per min. In our publication on kinetics of human 11β-HSD-II (10), we reported an incorrect unit, nmol/mg per min. In Table 1 of that paper, V_{max} units are also pmol/mg per min.

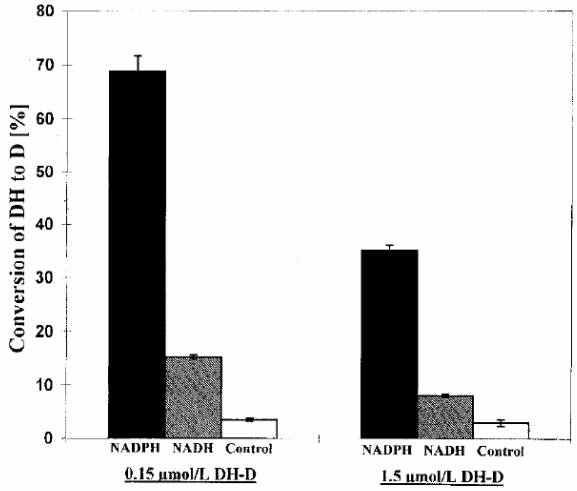

Figure 1 Cosubstrate dependence of the reduction of 11-dehydrodexamethasone (DH-D) to dexamethasone (D) in human liver microsomes. Percentage conversion was measured in the absence (Control) or in presence of 10^{-3} mol/l cosubstrate. Two different substrate concentrations (0.15 and 1.5 μmol/l DH-D) were tested. Protein concentration, 0.05 mg/ml. Incubation time, 30 min. Values are means ± S.D., each $n = 3$.

oxidation/reduction V_{max} ratio, $V_{max,ox}/V_{max,red}$), but oxidation of cortisol to cortisone is measurable. The K_m values for all reactions catalyzed by 11β-HSD-I are in the micromolar range (Table 2).

From the 15 potentially inhibitory substances of 11β-HSDs tested in concentrations from 10^{-9} to 10^{-5} mol/l, chenodeoxycholic acid (CDCA) was the only one that selectively inhibited 11β-HSD-I (Table 1, Fig. 2). In contrast, ketoconazole was a relatively selective inhibitor of 11β-HSD-II. Metyrapone has been reported to

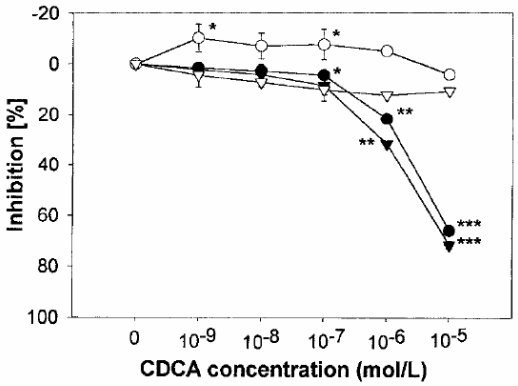

Figure 2 Percentage inhibition of 11β-HSDs by chenodeoxycholic acid (CDCA): ●, oxidation of cortisol (F) by 11β-HSD-I; ▼, reduction of 11-dehydro-dexamethasone (DH-D) by 11β-HSD-I; ○, oxidation of F by 11β-HSD-II; ▽, reduction of DH-D by 11β-HSD-II. The mean conversion in the absence of CDCA is defined as 100%. Values are means ± S.D. ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

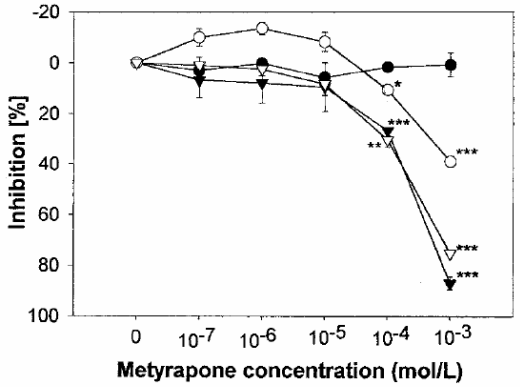

Figure 3 Percentage inhibition of 11β-HSDs by metyrapone: ●, oxidation of cortisol (F) by 11β-HSD-I; ▼, reduction of 11-dehydro-dexamethasone (DH-D) by 11β-HSD-I; ○, oxidation of F by 11β-HSD-II; ▽, reduction of DH-D by 11β-HSD-II. Mean conversion without metyrapone is defined as 100%. Values are means ± S.D. ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

preferentially inhibit 11β-HSD-I (26, 27). Since we only found weak inhibitory effects of metyrapone in concentrations between 10^{-9} and 10^{-5} mol/l, this substance was also tested at 10^{-4} and 10^{-3} mol/l. Metyrapone inhibited 11-keto-reductase reactions catalyzed by both isoenzymes and oxidative reaction of 11β-HSD-II, whereas the oxidation by 11β-HSD-I was not inhibited up to a concentration of 10^{-3} mol/l (Table 1, Fig. 3).

Discussion

Although the cDNA of human 11β-HSD-I (from a testis library) was isolated earlier (1991) than that of 11β-HSD-II (1994) (3, 4), the enzyme kinetics of the latter isoform have been better characterized in the human. One of the reasons for this was the new concept that the 11β-HSD-II protects the mineralocorticoid receptor from glucocorticoids and confers aldosterone specificity to this receptor. Kinetic studies of 11β-HSD-I were hampered by discrepancies between results obtained from examinations with intact cells and with cell lysates. In intact cells, 11β-reduction (e.g. of cortisone to cortisol) seems to be the preferred reaction (16–19). In homogenates or microsomes, however, demonstration of the reduction of 11-dehydrosteroids is more difficult than that of 11β-oxidation (28, 29). The published enzyme kinetic data for 11β-reduction by human 11β-HSD-I were, therefore, derived from studies with intact transfected cells, whereas data for 11β-oxidation were obtained in cell lysates (5, 29).

Our microsome preparations from human livers offered the opportunity to examine both catalytic reactions of 11β-HSD-I in the same approved system for enzyme kinetic studies. It is, therefore, not surprising that the results obtained differ somewhat from those of other workers. While Stewart *et al.* (29) found a higher

maximum velocity (V_{max}) for oxidation than reduction by a cloned type I isoenzyme, our findings of a higher V_{max} for the reductive reaction (Table 2) is more consistent with the concept of a mainly reducing 11β-HSD-I. The K_m value for the oxidation of F is about 2 times lower and the K_m value for the reduction of E is about 4 times higher than those in the study of Stewart *et al.* (29). Our results are, on the other hand, in agreement with other published findings (4, 29–31). i) The preferred cosubstrate is NADP or NADPH (Table 2, Fig. 1). ii) In contrast to 11β-HSD-II, neither the oxidative nor the reductive reaction of 11β-HSD-I is inhibited by reaction products (see Results, data not shown). iii) Dexamethasone is not a substrate for oxidation by 11β-HSD-I (Table 2). Whereas dexamethasone seems to have low or no affinity to the substrate binding site of 11β-HSD-I (30, 31), it increases the mRNA expression of 11β-HSD-I by interaction with the promoter region (18).

The reduction of DH-D (V_{max} = 741 pmol/mg per min) was faster than that of cortisone (V_{max} = 187 pmol/mg per min), similar to our findings with human kidney microsomes (10). In intact CHOP cells selectively transfected with cDNA of rat 11βHSD-I or -II (11), 11-dehydrodexamethasone (DH-D) had a higher affinity to the type I (K_m, 0.5 μmol/l) than the type II (K_m, 1.9 μmol/l) enzyme. The K_m of 1.6 μmol/l which we found for the reduction of DH-D by human 11β-HSD-I was higher than that reported by Li *et al.* (11) in transfected cells and that found in human renal microsomal 11β-HSD-II (K_m, 68 nmol/l) in our previous study (10). Altogether, our results fit well into the concept of a low affinity 11β-HSD-I and a high affinity 11β-HSD-II.

Although several reports on inhibitors of 11β-HSDs were published before, as yet, no selective inhibitor of one of the enzymes has been described. Recent *in vivo* studies suggest that selective blockade of the hepatic conversion of cortisone to cortisol could be beneficial in the therapy of diabetes mellitus with hepatic insulin resistance (decrease of gluconeogenesis) or in patients with central obesity (23). We decided to search for a selective inhibitor of hepatic 11β-reduction, because after blockade of 11β-HSD-I, the renal isoenzyme 11β-HSD-II could then selectively activate 11-dehydro-glucocorticoids like 11-dehydro-dexamethasone (DH-D) to form dexamethasone, with the aim of renal immunosuppression with minimal extrarenal side effects (renal targeting). For this reason, we studied a number of candidate compounds as inhibitors of the hepatic 11-oxidoreductase (Table 1). Most of the substances have never been tested in a comparative manner or for both reactions of the isoenzymes.

With regard to the 'classical' inhibitors of 11β-HSDs, glycyrrhetinic acid and carbenoxolone, we found only minor differences between these two inhibitors. Both inhibit the oxidative and reductive reactions at concentrations of 10^{-9} to 10^{-8} mol/l. These results are in variance from conclusions drawn from *in vivo* studies (32), but in agreement with more recently published *in vitro* results (33). 11α- and 11β-OH-progesterone are inhibitors of 11β-HSD-II (34), and enhance the mineralocorticoid activity of corticosterone *in vivo* (35). In our system, they are less potent as inhibitors of 11β-HSD-I than 11β-HSD-II, although they are not selective inhibitors. Other derivatives of progesterone and of deoxycorticosterone that were suggested to be selective inhibitors of one of the enzymes (36), were also unselective in our study (Table 1).

Detailed studies of the inhibitory effects of bile acids have been performed in rat renal microsomes, which mainly represent 11β-HSD-I activity (37). Chenodeoxycholic acid (CDCA) and lithocholic acid were the most potent inhibiting bile acids found in this study. Although CDCA had not shown any inhibitory effects on 11β-oxidation both in human renal cortical microsomes (38) and in a cell line with 11β-HSD-II activity (39), it fell into oblivion as a possible selective inhibitor of 11β-HSD-I. Our results clearly demonstrate (Table 1, Fig. 2) that CDCA is a selective inhibitor of human hepatic 11β-HSD-I. CDCA is being clinically used in the medical treatment of gallstones. Serum concentrations after oral ingestion of 250 mg and 750 mg CDCA reach 10^{-5} mol/l, and $>2\times10^{-5}$ mol/l, respectively (40). Since those concentrations clearly exceed the IC_{50} values for CDCA found in this study, and intrahepatic concentrations may even be higher than those in serum, it seems promising to perform adequate *in vivo* studies with this bile acid.

Metyrapone was reported to inhibit the 11β-HSD-I reductase activity (27). This was not an unexpected finding, since 11β-HSD-I is involved in the metabolism of metyrapone, reducing it to metyrapol (41). The inhibitory mechanism is, therefore, probably that of substrate competition. Metyrapone inhibited reductase activities of 11β-HSD-I and -II and the oxidase activity of 11β-HSD-II (Table 1, Fig. 3). The inhibition of steroid oxidation by 11β-HSD-II is probably due to the mechanism of 'product inhibition', a well known characteristic of this enzyme (4). Since the 11β-HSD-I oxidase activity is not inhibited by reaction products, metyrapone failed to inhibit this reaction, even at very high concentrations (Fig. 3).

The antifungal agent ketoconazole is a relatively selective inhibitor of 11β-HSD-II (Table 1). As oxidation by 11β-HSD-II is involved in local inactivation of glucocorticoids in target organs, this inhibition may be one explanation for the enhanced immunosuppressive effect of prednisolone when it is coadministered with ketoconazole (42).

We conclude that our microsome preparations from human liver and kidney cortex are a suitable model for comparing enzyme kinetics and inhibition characteristics of 11β-HSD-I and 11β-HSD-II. Since the affinity of 11-dehydro-dexamethasone to hepatic 11β-HSD-I is low, small oral doses of this substance may pass the liver

largely unmetabolized and may then be selectively activated by high affinity 11β-HSD-II in the kidney. This concept of renal glucocorticoid targeting by 9α-fluorinated 11-dehydrosteroids may be strengthened by oral coadministration of chenodeoxycholic acid, an obviously selective inhibitor of 11β-HSD-I. *In vivo* studies will be required to find out if this combination of drugs may be useful for immunosuppressive therapy targeted to specific organs harboring 11β-HSD-II and if therapy with chenodeoxycholic acid alone could be beneficial in patients with hepatic insulin resistance or central obesity.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DI741/1-1).

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Received 29 July 1999
Accepted 17 September 1999

3.1.4 AME Syndrom

4. **Quinkler M**, Bappal B, Draper N, Atterbury AJ, Lavery GG, Walker EA, DeSilva V, Taylor NF, Hala S, Rajendra N, Stewart PM.
   Molecular basis for the Apparent Mineralocorticoid Excess Syndrome in the Oman population.
   Mol.Cell.Endocrinol **2004,** 217: 143-149

ELSEVIER

Molecular and Cellular Endocrinology 217 (2004) 143–149

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Molecular basis for the Apparent Mineralocorticoid Excess Syndrome in the Oman population

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Abstract

11β-Hydroxysteroid dehydrogenase type 2 (11β-HSD2) plays a crucial role in converting hormonally active cortisol to inactive cortisone, thereby conferring specificity upon the mineralocorticoid receptor (MR). Mutations in the gene encoding 11β-HSD2 (HSD11B2) account for an inherited form of hypertension, the syndrome of "Apparent Mineralocorticoid Excess" (AME) where cortisol induces hypertension and hypokalaemia. We report five different mutations in the HSD11B2 gene in four families from Oman with a total of 9 affected children suffering from AME. Sequence data demonstrate the previously described L114Δ6nt mutation in exon 2 and new mutations in exon 3 (A221V), exon 5 (V322ins9nt) and for the first time in exon 1 (R74G and P75Δ1nt) of the HSD11B2 gene. These additional mutations provide further insight into AME and the function of the 11β-HSD2 enzyme. The prevalence of monogenic forms of hypertension such as AME remains uncertain. However, our data suggests AME may be a relevant cause of hypertension in certain ethnic groups, such as the Oman population.

Keywords: Hypertension; 11β-HSD2; AME syndrome; Hypokalaemia; Cortisol; Oman

1. Introduction

The mineralocorticoid receptor (MR) is non-selective in vitro and cannot distinguish between the glucocorticoid cortisol and its natural ligand, aldosterone (Arriza et al., 1987; Krozowski and Funder, 1983). Two isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) interconvert cortisol (F) and inactive cortisone (E). 11β-HSD2 is highly expressed in mineralococorticoid target tissues such as kidney, colon, and salivary glands and is co-localised with the MR. This enzyme functions to protect the MR by inactivating cortisol to cortisone thereby enabling aldosterone to occupy the MR in vivo (Edwards et al., 1988; Funder et al., 1988). Aldosterone is not metabolized by 11β-HSD2 because it forms a C_{11}–C_{18} hemi-ketal group in aqueous solution. Mutations in the gene encoding 11β-HSD2 (HSD11B2) explain the syndrome of Apparent Mineralocorticoid Excess (AME), an autosomal recessive inherited form of hypertension. This is characterised by hypertension and hypokalaemia with suppression of plasma renin and aldosterone concentrations (New et al., 1977; Quinkler and Stewart, 2003; Stewart and Krozowski, 1999; Ulick et al., 1977; Werder et al., 1974; White et al., 1997). It is caused by a defective 11β-HSD2 allowing cortisol, which has higher circulating levels than aldosterone, to bind to the MR and to act as a potent mineralocorticoid. Patients are diagnosed by a raised tetrahydro(TH)F + allo-THF/THE urinary ratio.

The human HSD11B2 is 6.2 kb long, comprising five exons and is located on chromosome 16q22 (Agarwal et al., 1995). Less than 100 cases of AME have been reported and over 30 different mutations defined within exons 2–5 of HSD11B2 in affected cases. Data from our own group, and others, have highlighted a close correlation between disease phenotype and genotype (Nunez et al., 1999; Stewart et al., 1996; White et al., 1997). Recently, nine patients with AME from four unrelated families from Oman have been referred to us for genetic studies, and here we describe the molecular basis for AME within these kindreds.

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doi:10.1016/j.mce.2003.10.019

2. Materials and methods

2.1. Patients and clinical characteristics

The patients are derived from four different families from Oman (Fig. 1). The families all have pure Omani ethnicity. The clinical characteristics and biochemical features of patients with AME are presented in Table 1. The male to female ratio was 5:4. Nearly all affected patients showed severe intrauterine growth retardation with a low birthweight compared to their unaffected siblings. Nearly all patients presented at a very young age (0.2–3.8 years) with failure to thrive (poor weight gain), hypokalemic metabolic alkalosis and significant hypertension compared to the 90th percentile for normal age- and sex-matched children. The majority of the patients had polyuria and polydipsia as a consistent presenting feature in early childhood presumably due to nephrogenic diabetes insipidus secondary to hypokalemic nephropathy. Other causes of endocrine hypertension, such as primary hyperaldosteronism, dexamethasone suppressible hyperaldosteronism, Liddle's syndrome and congenital adrenal hyperplasia were excluded by hormonal studies. The urinary THF + allo-THF/THE ratio ranged from 5.2 to 50.3. All patients had a high urinary allo-THF/THF ratio (range 1.7–5.0) suggesting an additional defect in 5β-reductase activity as described in previous cases (Monder et al., 1986). After diagnosis some patients were treated with dexamethasone and potassium chloride supplementation, but in the follow-up, all patients were changed to treatment with amiloride and/or spironolactone. In some cases further anti-hypertensive drugs, such as nifedipine, were added, as well as potassium chloride supplementation.

2.2. Laboratory methods and procedures

Twenty-four hours urine samples were collected and urinary steroid metabolites were measured as reported (Raven et al., 1995; Raven and Taylor, 1996). Blood samples for steroids and electrolyte determinations were reported at baseline while patients were receiving a normal sodium diet.

2.3. DNA sequencing of HSD11B2

Genomic DNA was recovered from peripheral blood leucocytes from affected kindreds. PCR was used to amplify overlapping fragments of HSD11B2 (excluding the majority of intron 1) using primer sequences previously described (Lavery et al., 2003). PCR reactions were carried out in a

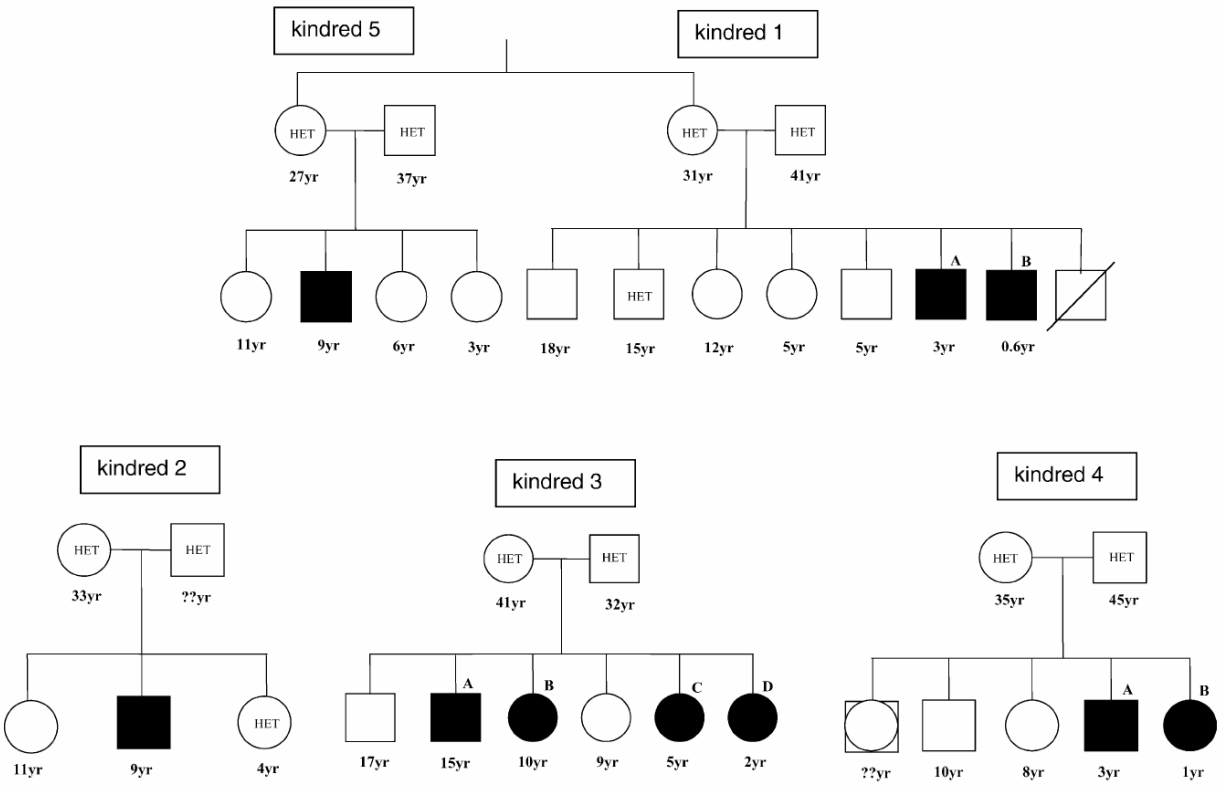

Fig. 1. Pedigree of AME kindreds in four families from Oman (black = affected and homozygous for mutations, white = unaffected wild type or heterozygous). The sex of one child in kindred 4 is not known (circle and square). One boy in kindred 1 died at the age of 1 year (square with crossed line). The diagnosis of AME in patient B of kindred 1 was made clinically, and genetic testing has yet to be performed.

Table 1
Clinical characteristics and biochemical features of patients with AME at presentation

Patient	Age (year)	Sex	Birth weight (kg)	Hx of FTT	Polyuria/ polydipsia	BP (mmHg)	BP (90th centile for age)	Serum K^+ (mmol/l)	Serum Na^+ (mmol/l)	pH/HCO_3^- (mmol/l)/BE	(THF + 5αTHF)/ THE	5αTHF/ THF
Kindred 1												
A	1.7	M	2.7	Y	Y	155/71	106/71	1.5	137	7.48/33/+8.6	6.2	3.1
B	0.3	M	2.2	NA	NA	121/78	105/67	2.7	137	7.46/30/+6.8	NA	NA
Kindred 2	2.0	M	1.7	Y	Y	132/96	92/66	1.5	138	7.43/30/+4.5	16.3	3.2
Kindred 3												
A	3.8	M	1.8	Y	Y	169/101	108/69	2.5	145	7.46/39/+12.6	5.2	2.0
B	1.0	F	2.1	Y	Y	140/90	108/67	2.9	143	7.51/34/+11	8.3	1.7
C	0.2	F	1.9	NA	NA	124/87	98/65	4.2	139	7.34/23/+0.8	6.6	1.8
D	0.7	F	2.2	Y	Y	103/53	102/68	2.4	137	NA	9.3	4.5
Kindred 4												
A	1.8	M	1.6	Y	Y	148/88	106/68	2.1	152	7.41/28/+2.3	13.6	3.2
B	1.0	F	1.9	NA	NA	127/72	105/67	2.5	136	NA/27/NA	NA	NA
Kindred 5	1.5	M	2.1	NA	Y	146/94	106/68	2.6	145	7.49/NA/+5.9	50.3	5.0

The age indicated is the age when the patient first came to medical attention. The diagnosis of AME in patient B of kindred 1 was made clinically, and genetic testing has yet to be performed. Hx of FTT: history of failure to thrive; BP: blood pressure; BE: base excess; N: no; Y: yes; NA: not available. Normal K^+ levels: 3.5–4.5 mmol/l. Normal urinary (THF + 5α-THF)/THE ratio: 0.7–1.2. Normal urinary 5α-THF/THF ratio: 0.7–1.4.

25 μl reaction volume containing 100–200 ng of genomic DNA, 25 pmol of each primer, 1x PCR buffer and 2U of Proofstart DNA polymerase (Qiagen, Crawley, UK). All PCR reactions were carried out in a PE9700 thermal cycler (Perkin-Elmer, Foster City, USA) using the following cycle conditions: an initial denaturation step of 95 °C × 5 min, followed by 32 cycles of 95 °C × 25 s, 55 °C × 25 s and 72 °C × 25 s. The amplified DNA products were recovered after gel electrophoresis and directly sequenced using an ABI prism 377 DNA sequencer (Perkin-Elmer, Foster City, USA).

Sequences were compared to HSD11B2 genomic sequence (Genbank accession number: U27317).

3. Results

3.1. Clinical and biochemical features and complications

The biochemical features of our AME patients at presentation are shown in Table 1. End-organ damage was observed only in a few patients (Table 2). Five of six (88%) patients had hypercalciuria, indicated by an elevated urinary calcium/creatinine molar ratio ranging from 0.31 to 1.6. Nephrocalcinosis was found in 3 of 5 (60%) patients. Hypertensive retinopathy was not reported in any of our cases. Echocardiography revealed left ventricular hypertrophy in five of eight (62%) patients. Two patients had to be treated temporarily on the intensive care unit due to cardiac arrest as a result of hypokalemia and due to respiratory insufficiency caused by muscular weakness. A third child was admitted because of congestive heart failure. In the family of kindred 3, eight pregnancies were reported with two conceptions ending in abortion. In addition, two of these pregnancies were complicated by mild polyhydramnions in the last two affected siblings. In the family of kindred 1, one boy died at the age of 1 due to acute heart failure (Fig. 1).

3.2. Mutations in HSD11B2

In kindred 1 and 5, homozygosity for a novel non-conservative missense mutation, R74G, was detected in exon 1 in conjunction with homozygosity for a novel mutation, P75Δ1nt, which caused a shift (−1) in the exon 1 reading frame. These are the first mutations to be reported in exon 1 of HSD11B2. These mutations are predicted to generate a stop codon, leading to a truncated protein, and no 11β-HSD2 enzyme activity.

In kindred 2, homozygosity for a six-nucleotide deletion in exon 2 was detected. The mutation, L114Δ6nt, causes the loss of Leu114 and Glu115 form the 11β-HSD2 protein. Previously these residues have been shown to be crucial for cofactor binding and are therefore likely to result in attenuated enzyme activity (Odermatt et al., 2001).

In kindred 3, homozygosity for a novel missense mutation, A221V, was detected in exon 3. We speculate that pre-mRNA splicing may be compromised as the mutation lies two base pairs from the intron 3 donor splice site.

In kindred 4, homozygosity for a novel nine nucleotide insertion was discovered in exon 5. This mutation, V322ins9nt, maintains the correct reading frame whilst inserting three additional codons encoding, alanine, proline and valine. This mutation may lead to defective secondary structure formation, and would be predicted to severely attenuate 11β-HSD2 activity.

Fig. 2 shows the mutations identified in each kindred and Fig. 3 shows the sequencing electropherograms obtained for each mutation in the homozygous state.

Table 2
Complications (end-organ damage) in AME patients

Patient	Urinary Ca/Cr	Nephrocalcinosis	LVH	Other complications
Kindred 1				
A	1.1–1.6	Y	NA	–
B	NA	NA	Mild	Congestive heart failure (age 0.5)
Kindred 2	0.88	Y (age 5)	N	Cardiac arrest (age 3.5)
Kindred 3				
A	0.31	N	Mild	Respiratory failure (age 4.2)
B	0.77	N	N	–
C	0.92	Y	N	–
D	NA	N	NA	–
Kindred 4				
A	NA	NA	Mild	–
B	NA	NA	Mild	–
Kindred 5	<0.2	N	Mild	–

The diagnosis of AME in patient B of kindred 1 was made clinically, and genetic testing has yet to be performed. Urinary Ca/Cr: calcium/creatinine molar ratio (normal <0.2). LVH: left ventricular hypertrophy; N: no; Y: yes; NA: not available.

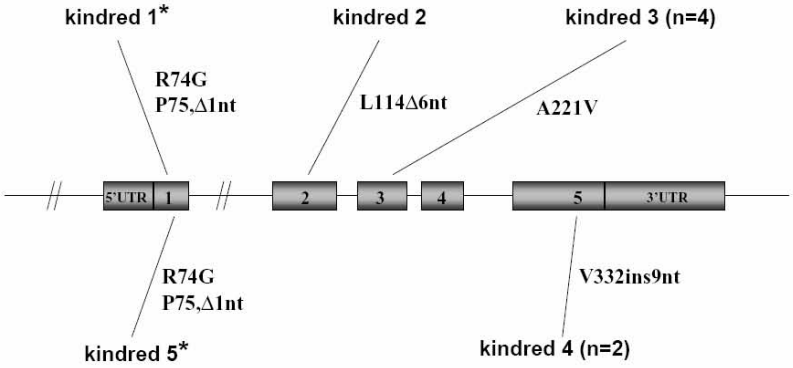

Fig. 2. Mutations in HSD11B2 causing AME in the Oman population. (*) Kindred 1 and 5 are cousins (their mothers are sisters).

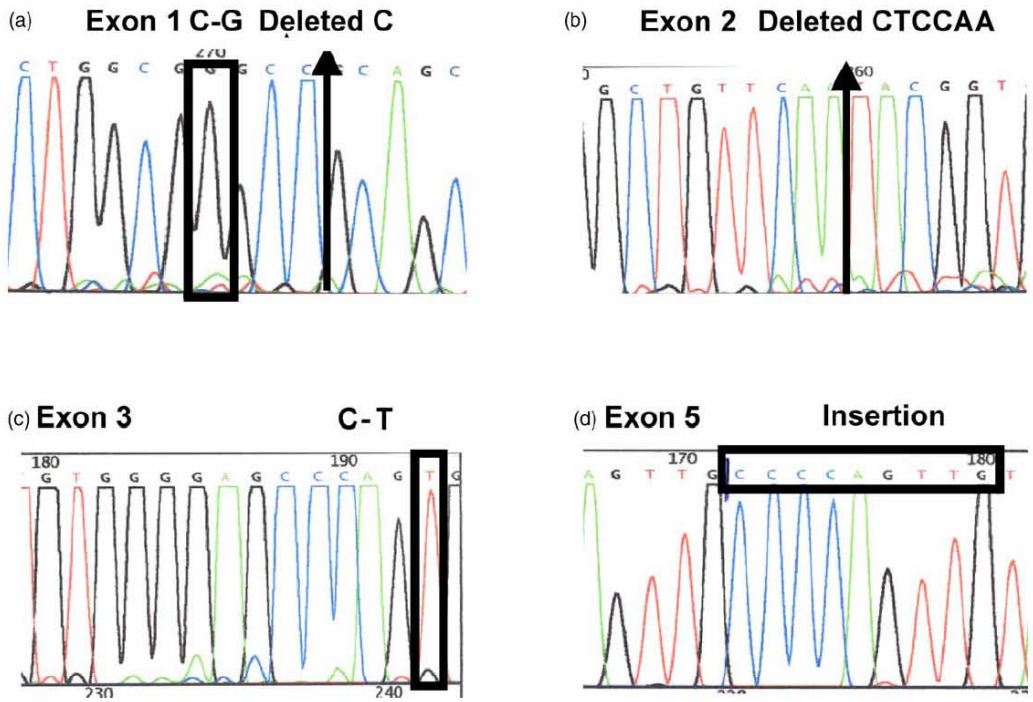

Fig. 3. Sequencing electropherograms of the mutations in the homozygous state within exons 1–3 and 5: (a) kindred 1 and 5; (b) kindred 2; (c) kindred 3; (d) kindred 4.

4. Discussion

The syndrome of AME is an unusual form of hypertension that has defined an important "pre-receptor" pathway in the analysis of corticosteroid hormone action. 11β-HSD2 serves to protect the MR from glucocorticoid excess through its inactivation of cortisol to cortisone. Due to a defective 11β-HSD2 enzyme in AME patients, urinary steroid metabolite profiles show elevated cortisol metabolites and very low or absent levels of cortisone metabolites. Therefore the THF + allo-THF/THE ratio has historically been used in the diagnosis of AME (Monder et al., 1986; Shackleton et al., 1985). Most AME patients are homozygous for HSD11B2 mutations resulting in full, or partial loss of activity. However, a close correlation is reported between disease phenotype (as measured by the THF + allo-THF/THE ratio, serum potassium and blood pressure) and genotype (Nunez et al., 1999). Patients with mutant 11β-HSD2 cDNA's that demonstrate little or no activity in vitro, present in early life with severe, often life-threatening, hypertension and hypokalaemia. In contrast, patients presenting in late adolescence or early adulthood with so-called "mild" forms of AME (earlier referred to as AME type II) have been found to have mutations

that result in an 11β-HSD2 protein with only attenuated activity.

Here we report nine patients with AME from four families from Oman. All patients presented in their first 4 years of life with life threatening hypokalaemia and hypertension. The early onset and the severe symptoms suggest that the mutations in these patients result in a complete loss of 11β-HSD2 activity. Five mutations, four novel and the previously reported L114Δ6nt (Odermatt et al., 2001), were identified by genomic analysis. They are predicted to either abolish or severely attenuate enzyme activity. We report for the first time two mutations within exon 1 of HSD11B2 leading to the AME phenotype. With the addition of this study, mutations that explain the molecular basis for AME have been identified in all exons and two introns of the HSD11B2 gene.

AME is most commonly found in consanguineous families (Mune et al., 1995; Stewart et al., 1996; White et al., 1997; Wilson et al., 1995a,b). Homozygosity in AME is thought to result from endogamy or a founder effect in Native American families with the R208C and E356Δ1nt mutations, and the L250S, L251P mutation (Mune et al., 1995; Wilson et al., 1995a). The fact that six kindreds are of Native American origin has prompted speculation as to a possible selective advantage of heterozygotes. Such individuals may have an increased ability to conserve salt under conditions of extreme sodium deprivation (White et al., 1997).

The Oman population comprises 2.2 million people with a majority (73%) of Omanis and minorities of Indians, Pakistanis and East Africans. The population growth is high (3.71% per year) and the age structure is very young (46% are between 0 and 14 years). The population is described as quite heterogeneous due to an ethic and religious mix derived mainly from a history of maritime trade and tribal migration. Nevertheless, the tribal customs consist of a strong preference for marriages between the son and his paternal uncle's daughter or with a first cousin on the mother's side, or with a more distant cousin. Marriage outside the extended family is described as exception, but does also occur. In 1995, Wilson et al. (1995a) described a family from Oman with two boys having AME syndrome. Their parents and three other siblings were heterozygous for a R208C mutation. The authors suggested that the homozygosity of this rare mutation in these kindreds was explained by endogamy, because the family derived from the Mozaini tribe, where consanguinity and intra-tribal marriages are customary.

Our cases all have pure Omani ethnicity and derive from four different tribes. The parents of kindred 1–3 and the grandparents of kindred 3 are second degree relatives. This suggests that the homozygosity of those mutations may be explained by consanguinity in the tribal society. Therefore, the diagnosis of AME should be suspected in patients from tribal societies who present with the features of low birth weight, failure to thrive, polyuria and polydipsia, and hypokalaemia and hypertension. The baseline evaluation should consist of determination of plasma renin and aldosterone, which are both suppressed in AME. The next diagnostic step should be the determination of urinary cortisol metabolites and the diagnosis is confirmed by DNA analysis (Quinkler and Stewart, 2003).

In conclusion, these additional mutations provide further insight into AME and the function of the 11β-HSD2 enzyme. The prevalence of monogenic forms of hypertension such as AME remains uncertain. However, our data suggests AME may be a common cause of hypertension in certain ethnic groups, such as the Oman population.

Acknowledgements

We would like to thank Dr. P.J. Wood, Regional Endocrine Laboratory, Department of Chemical Pathology, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, UK for his help with urinary cortisol and cortisone metabolite measurements. This work was supported by a Deutsche Forschungsgemeinschaft (DFG) postdoctoral research fellowship grant (QU142/1-1) to MQ and the Medical Research Council (senior clinical fellowship to PMS).

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3.1.5 Ektopes ACTH Syndrom

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© European Journal of Endocrinology 1996, 134: 301–7

Human kidney 11β-hydroxysteroid dehydrogenase: regulation by adrenocorticotropin?

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Diederich S, Quinkler M, Miller K, Heilmann P, Schöneshöfer M, Oelkers W. Human kidney 11β-hydroxysteroid dehydrogenase: regulation by adrenocorticotropin? Eur J Endocrinol 1996;134:301–7. ISSN 0804–4643

In ectopic adrenocorticotropin (ACTH) syndrome (EAS) with higher ACTH levels than in pituitary Cushing's syndrome and during ACTH infusion, the ratio of cortisol to cortisone in plasma and urine is increased, suggesting inhibition of renal 11β-hydroxysteroid dehydrogenase (11β-HSD) by ACTH or by ACTH-dependent steroids. Measuring the conversion of cortisol to cortisone by human kidney slices under different conditions, we tested the possibility of 11β-HSD regulation by ACTH and corticosteroids. Slices prepared from unaffected parts of kidneys removed because of renal cell carcinoma were incubated with unlabeled or labeled cortisol, and cortisol and cortisone were quantitated after HPLC separation by UV or radioactive detection. The 11β-HSD activity was not influenced by incubation with increasing concentrations (10^{-12}–10^{-9} mol/l) of ACTH (1–24 or 1–39) for 1 h. Among 12 ACTH-dependent steroids tested (10^{-9}–10^{-6} mol/l), only corticosterone ($IC_{50} = 2 \times 10^{-7}$ mol/l), 18-OH-corticosterone and 11βOH-androstenedione showed a significant dose-dependent inhibition of 11β-HSD activity. The percentage conversion rate of cortisol to cortisone was concentration dependent over the whole range of cortisol concentrations tested (10^{-8}–10^{-5} mol/l). A direct inhibitory effect of ACTH on 11β-HSD is, therefore, unlikely. The only steroids inhibiting the conversion of cortisol to cortisone are natural substrates for 11β-HSD. Kinetic studies show a saturation of the enzyme at high cortisol concentrations. Thus, the reduced percentage renal cortisol inactivation in EAS seems to be due mainly to overload of the enzyme with endogenous substrates (cortisol, corticosterone and others) rather than to direct inhibition of 11β-HSD by ACTH or ACTH-dependent steroids, not being substrates of 11β-HSD.

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Purified or recombinant mineralocorticoid receptors (type I corticosteroid receptors) are non-selective in vitro, and bind cortisol, corticosterone and aldosterone with nearly similar affinity (1, 2). Aldosterone selectivity in mineralocorticoid target tissues is mainly due to 11β-hydroxysteroid dehydrogenase (11β-HSD), an enzyme that catalyzes the oxidation of endogenous glucocorticoids (cortisol in man and corticosterone in the rat) to their biologically inactive keto products (3, 4). This mechanism allows selective access of aldosterone to renal mineralocorticoid receptors in vivo, despite a 100-fold molar excess of circulating free glucocorticoids. Congenital abnormalities of 11β-HSD (the syndrome of apparent mineralocorticoid excess (AME)) and acquired inhibition of 11β-HSD by licorice and carbenoxolone ingestion result in cortisol and corticosterone acting as potent mineralocorticoids (5).

Recently the existence of isoenzymes of 11β-HSD has been shown (6). There is now strong evidence that rat (7), rabbit (8), ovine (9) and human (10, 11) kidneys possess a NAD-dependent isoform (type 2) that is "optimized" for the protection of mineralocorticoid receptors (high affinity, only oxidative activity).

The ectopic ACTH syndrome (EAS) can be distinguished from other causes of Cushing's syndrome by more severe symptoms of mineralocorticoid excess, e.g. hypokalemic alkalosis, and higher plasma levels of cortisol (12, 13). In EAS with higher ACTH levels than in pituitary Cushing's syndrome, the ratio of cortisol to cortisone in plasma (14) and urine (16) is increased, suggesting inhibition of renal 11β-HSD by ACTH or by ACTH-dependent steroids. Walker et al. (14) and Eisenschmid et al. (15) observed a marked increase of the cortisol/cortisone ratio in urine or plasma during ACTH infusion, but not following hydrocortisone infusion in man. This led to the conclusion that ACTH directly or indirectly inhibits renal 11β-HSD (14), while Ulick et al. (16) favor saturation of the enzyme by cortisol excess per se as the cause of a high cortisol/cortisone ratio in high ACTH states.

By measuring the effect of ACTH and different corticosteroids on the conversion of cortisol to cortisone

in an in vitro model with human kidney slices (17), we evaluated the different hypotheses of 11β-HSD regulation in states of ACTH excess.

Materials and methods

Materials used

Cortisol, aldosterone, corticosterone, 18-OH-corticosterone, 17α-OH-progesterone, androstenedione, 11β-OH-androstenedione and 11-deoxycortisol were obtained from Sigma Chemical Co. (St Louis, MO). 17,20α-OH-Progesterone and 18-OH-deoxycorticosterone (18-OH-DOC) were purchased from Steraloids Inc. (Wilton, NH) and progesterone and DOC from Serva Feinbiochemica (Heidelberg/New York). 18β-Glycyrrhetinic acid was obtained from Aldrich-Chemie (Steinheim, Germany), DHEA from Paesel & Lorei GmbH & Co. (Frankfurt, Germany), $ACTH_{(1-39)}$ from Peninsula Laboratories GmbH (Heidelberg, Germany) and $ACTH_{(1-24)}$ (Synacthen®) from Ciba-Geigy GmbH (Wehr, Germany). (1,2,6,7-[^{3}H](N)-Cortisol (specific activity 70.0 Ci/mmol) was purchased from DuPont de Nemours GmbH (Bad Homburg v.d.H., Germany) and purified by preparative high-performance liquid chromatography prior to use.

We used the following solvents for sample clean-up and chromatography: acetone, *n*-hexane and 2-propanol (all LiChrosolv quality) were purchased from Merck Ltd. (Darmstadt, Germany) and methanol (LiChrosolv quality) from JT Baker B.V. (Deventer, The Netherlands). The Sep-Pak C_{18} cartridges were obtained from Waters Millipore GmbH (Eschborn, Germany), the LiChrosorb Diol reversed-phase high-pressure liquid chromatography (HPLC) columm (particle size 5 μm) and the reversed-phase precolumn (LiChrosorb Diol, particle size 5 μm) were from VDS optilab (Berlin, Germany) and the radioactivity scintillant Ultima Gold® was from Packard Instruments B.V. (Groningen, The Netherlands).

Krebs–Ringer bicarbonate (KRB) buffer consisted of 140 mmol/l Na^+, 4 mmol/l K^+, 2.5 mmol/l Ca^{2+}, 1.2 mmol/l Mg^{2+}, 123 mmol/l Cl^-, 1.2 mmol/l PO_4^{3-}, 1.2 mmol/l SO_4^{3-}, 24 mmol/l HCO^{3-} and 5.5 mmol/l glucose.

In vitro studies

Slices prepared from unaffected parts of kidneys removed because of renal cell carcinoma were incubated with unlabeled or labeled cortisol, and cortisol and cortisone were quantitated after HPLC separation by UV or radioactive detection. The preparation and incubation of human kidney slices was described previously (17); for the incubations we only used renal cortex.

Experiment 1. The possibility of a direct effect of ACTH on renal 11β-HSD was tested by measuring the conversion of unlabeled cortisol (10^{-7} mol/l) with increasing concentrations of $ACTH_{(1-24)}$ [Synacthen®] or $ACTH_{(1-39)}$ (10^{-12}–10^{-9} mol/l). Using two kidneys, five independent incubations for each ACTH concentration were performed (two with kidney 1; three with kidney 2). After incubating for 60 min, cortisol and cortisone were quantitated by UV absorption (17).

Experiment 2. We tested the inhibitory effect on renal 11β-HSD of the following ACTH-dependent steroids: corticosterone, 11-deoxycortisol, DOC, 18-OH-corticosterone, 18-OH-DOC, dehydroepiandrosterone (DHEA), androstenedione, 11β-OH-androstenedione, progesterone, 17-OH-progesterone, 17,20-OH-progesterone and aldosterone. The steroids selected are increased significantly following ACTH administration to normal volunteers (18–23) and/or in patients with endogenous ACTH oversecretion (e.g. ectopic or pituitary Cushing's syndrome) (14, 24–26). The oxidative activity of 11β-HSD was determined by measuring the conversion of 100 000 cpm (= 10^{-9} mol/l) [^{3}H]cortisol to [^{3}H]cortisone in the presence of 10^{-8} mol/l unlabeled cortisol. This would be a low–normal "free" plasma cortisol concentration (27). The steroids tested (10^{-9}–10^{-6} mol/l) were added to the reaction mixture. For each steroid and each concentration, five independent incubations were performed. Incubations with increasing concentrations of glycyrrhetinic acid (10^{-7}–10^{-5} mol/l; $n = 4$ for each concentration), a known inhibitor of 11-HSD, were also performed.

Experiment 3. The concentration dependence of renal 11β-oxidation was studied by measuring the conversion of 100 000 cpm (= 10^{-9} mol/l) [^{3}H]cortisol to [^{3}H]cortisone in the presence of increasing concentrations of unlabeled cortisol (10^{-8}–10^{-5} mol/l; at least three independent incubations for each concentration).

For experiments 2 and 3, two kidneys each were used; the incubation time for both experiments was 90 min.

Analytical procedure

Experiment 1. In the samples from this experiment, the 11-hydroxy- and the 11-oxo-steroids were separated by a reversed-phase HPLC and quantified by UV detection (248 nm wavelength). Details of the fully automated procedure have been described previously (17, 28).

Experiments 2 and 3. In order to precipitate the proteins, 2 ml of methanol was added to each sample, which was then stirred on a vortex mixer for 20 s and centrifugated for 10 min at 3000 rpm and 1°C. The liquid phase was pipetted into a tube with 6 ml of water and put on the vortex for 10 s. The samples were then poured slowly through Sep-Pak C_{18} cartridges that were activated before use with 5 ml of methanol and 5 ml of water (29).

The samples were washed successively with 2 ml of water, 1 ml of 10% methanol in water and 2 ml of 20%

acetone in water. The steroids were eluted with 3 ml of methanol from the cartridges. The methanol was then evaporated and the tubes were stored at −20°C.

For ^{3}H measurement, 200 μl of solvent (75% hexane and 25% 2-propanol) was pipetted into each tube, put on the vortex for 10 s and then centrifuged for 5 min at 3000 rpm and 1°C. Then 100 μl of the sample was injected into the reversed-phase HPLC column that was run with an isocratic solvent mixture (80% hexane and 20% 2-propanol; flow rate 1.3 ml/min) for 12 min. For the detection of radioactivity, liquid scintillant was added at a flow rate of 4 ml/min and the radioactivity was measured with a Berthold HPLC radioactivity monitor (model LB 506 C1, Berthold GmbH & Co, Wildbad, Germany). Ultraviolet absorption at a wavelength of 248 nm was also measured using a Bischoff Lambda 1000 instrument (Bischoff GmbH, Leonberg, Germany) as a control for the retention times of the steroids. The cortisol and cortisone peaks were always clearly separated. Blank incubates without tissue slices never exhibited metabolism of the substrate steroid. The conversion of 11-hydroxysteroids by tissue slices was expressed as a percentage of the total steroid measured.

Statistical calculations were done with an SPSS program from SPSS Inc. (Chicago, IL); the Duncan multiple range test was used.

Results

In human kidney slices, the 11β-oxidation of cortisol is not influenced by incubation with increasing doses of physiological $ACTH_{(1-39)}$ for 1 h (Fig. 1a). The lowest tested dose (10^{-12} mol/l) of synthetic $ACTH_{(1-24)}$ showed a slightly inhibiting effect on 11β-HSD activity, whereas the highest tested dose (10^{-9} mol/l) was slightly stimulating (Fig. 1b). As the changes in 11β-HSD activity are relatively small (but significant) and no concentration dependence was seen, a relevant direct effect of synthetic ACTH on renal 11β-oxidation seems to be unlikely.

Among the 12 ACTH-dependent steroids tested, only corticosterone, 18-OH-corticosterone and 11β-OH-androstendione showed a dose-dependent inhibition of the renal conversion of cortisol to cortisone (Table 1 and Fig. 2). The conversion rate in the absence of the inhibitors is defined as 100%. The IC_{50} (concentration at 50% inhibition) for corticosterone was 2×10^{-7} mol/l. For 11β-OH-androstenedione and 18-OH-corticosterone with a lower inhibiting potency, we could not calculate the IC_{50} because the steroid concentrations (10^{-9}–10^{-6} mol/l) studied were only in a clinically relevant and not in a highly supraphysiological range (27). No other corticosteroid tested inhibited the renal 11β-HSD in the concentrations used. Figure 3 shows the inhibition of the conversion of cortisol to cortisone by kidney slices in the presence of glycyrrhetinic acid. The IC_{50} is near 10^{-6} mol/l.

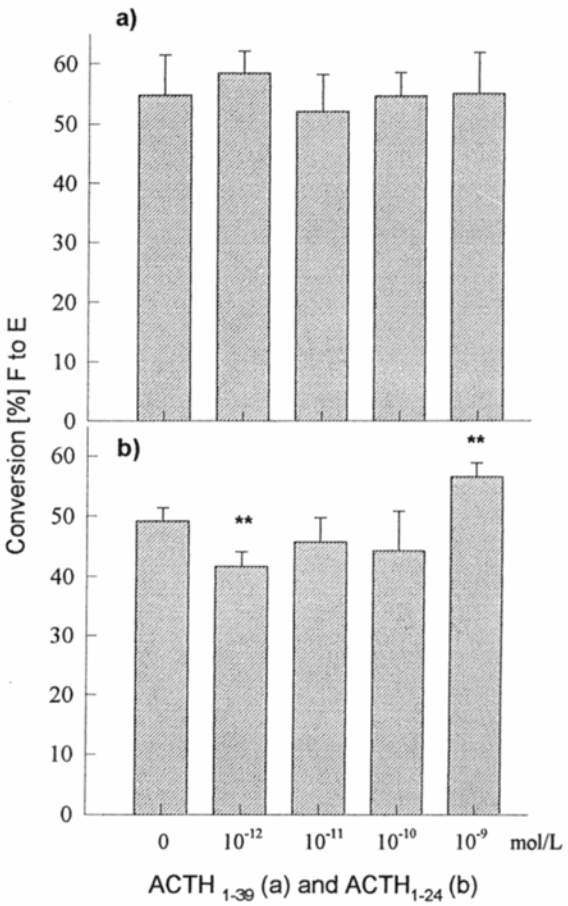

Fig. 1. Percentage conversion of cortisol (F, 10^{-7} mol/l) to cortisone (E) by kidney slices in the presence of increasing concentrations of: (a) $ACTH_{(1-39)}$; (b) $ACTH_{(1-24)}$. Values are means ± SEM (N = 5). **p < 0.01.

Table 1. Effects of 12 ACTH-dependent steroids on the conversion of cortisol (10^{-8} mol/l) to cortisone in human kidney slices.[a]

ACTH-dependent steroid	Concentration of significant 11β-HSD-inhibition
Corticosterone	10^{-7} mol/l, $IC_{50} = 2 \times 10^{-7}$ mol/l
18-OH-Corticosterone	10^{-6} mol/l
11-OH-Androstenedione	10^{-6} mol/l
Androstenedione	No inhibition
11-Deoxycortisol	No inhibition
Deoxycorticosterone	No inhibition
18-OH-Deoxycorticosterone	No inhibition
Dehydroepiandrosterone	No inhibition
Progesterone	No inhibition
17-OH-Progesterone	No inhibition
17,20-OH-Progesterone	No inhibition
Aldosterone	No inhibition

[a] No inhibition means no effect on 11β-hydroxysteroid dehydrogenase (11β-HSD) activity in the range of concentrations studied (10^{-9}–10^{-6} mol/l).

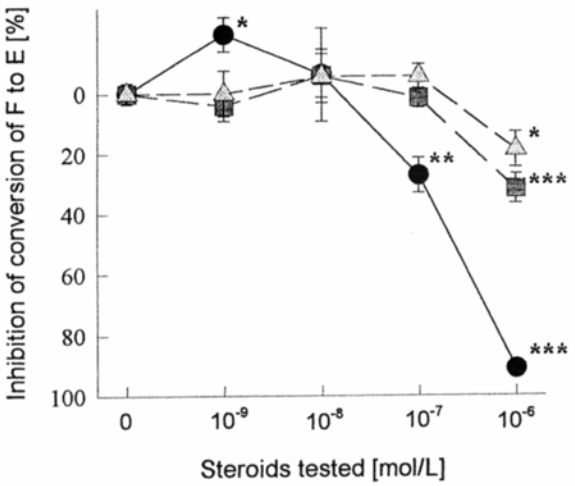

Fig. 2. Inhibition of 11β-oxidation of cortisol (F, 1.1×10^{-8} mol/l) to cortisone (E) in kidney slices by increasing concentrations of corticosterone (●), 18-OH-corticosterone (△) and 11β-OH-androstenedione (□). The mean conversion without inhibiting steroids for both kidneys used was $32.75 \pm 1.8\%$ and is defined as 100%. Values are means ± SEM (N= 5); *p < 0.05, **p < 0.01 and ***p < 0.001.

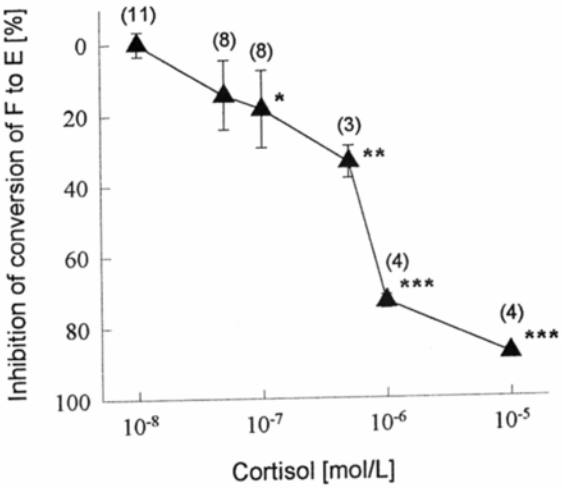

Fig. 4. Inhibition of the conversion of 10^{-8} mol/l [^{3}H] cortisol to [^{3}H] cortisone by increasing concentrations of unlabeled cortisol in human kidney slices. The mean conversion at 10^{-8} mol/l for both kidneys used was $39.4 \pm 3\%$ and is defined as 100%. Means ± SEM are shown; numbers of independent incubations are shown in parentheses. *p < 0.05, **p < 0.01 and ***p < 0.001.

Figure 4 shows the percentage conversion rate of [^{3}H] cortisol to [^{3}H] cortisone as a function of unlabeled cortisol concentrations (10^{-8}–10^{-5} mol/l). The conversion was concentration dependent over the whole range of concentrations tested. The conversion rate of 10^{-9} mol/l [^{3}H] cortisol in the presence of 10^{-8} mol/l unlabeled cortisol is defined as 100%.

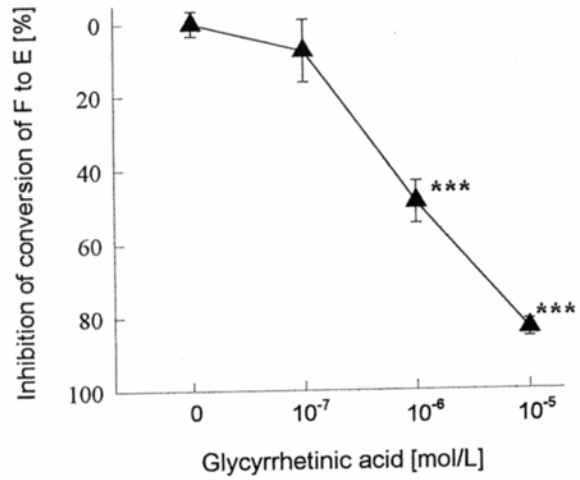

Fig. 3. Inhibition of the conversion of cortisol (F, 1.1×10^{-8} mol/l) to cortisone (E) by kidney slices in the presence of glycyrrhetinic acid. The mean conversion without inhibitor was $32.75 \pm 1.8\%$ and is defined as 100%. Values are means ± SEM (N= 4); ***p < 0.001.

Discussion

Cortisol can act as a potent mineralocorticoid if its renal inactivation by 11β-HSD is decreased (3–5). The increased cortisol/cortisone ratio during ACTH infusion (14, 15) suggests that this mechanism also operates in the mineralocorticoid manifestation of EAS. Based on a study of the urinary steroid metabolite profiles of two patients with EAS, Ulick et al. (16) assumed saturation of 11β-HSD by very high circulating cortisol concentrations as a cause of the high urinary cortisol/cortisone ratio in these patients. Walker et al. (14), however, found a higher plasma cortisol/cortisone ratio during ACTH infusion than during cortisol infusion into dexamethasone-suppressed normal volunteers, thus giving evidence for 11β-HSD inhibition by ACTH itself or by ACTH-dependent steroids other than cortisol. The comparability of cortisol levels obtained in Walker's study (14) by the two types of infusion, however, is not quite convincing.

Our in vitro studies allow some conclusions as to the effects of ACTH and cortisol on renal 11β-HSD.

The results of the incubation of human kidney slices with increasing doses of ACTH (Experiment 1, Fig. 1a,b) speak against a direct inhibition of renal 11βHSD by ACTH. The tested ACTH concentrations include the physiological and pathophysiological relevant range (plasma ACTH in normal subjects: 10^{-12}–10^{-11} mol/l; plasma ACTH in patients with EAS: 10^{-11}–10^{-10} mol/l) (12, 14). We cannot exclude a long-term direct effect of ACTH on renal 11β-HSD. The in vivo studies mentioned in the introduction (14, 15) show a very fast effect of

ACTH infusion on the cortisol/cortisone ratio; at least this fast effect does not seem to be mediated by direct inhibition of renal 11β-HSD. Apart from our results, other facts speak against a direct effect of ACTH on renal 11β-HSD: the recently cloned ACTH receptor is not expressed in the kidney (30) and ACTH treatment has no measurable effect on in vitro 11β-HSD activity in adrenalectomized rats (31).

Among the 12 tested ACTH-dependent corticosteroids (18–26), only corticosterone, 18-OH-corticosterone and 11β-OH-androstenedione inhibited the renal conversion of cortisol to cortisone in a dose-dependent manner (Table 1 and Fig. 2). These compounds are natural substrates of 11β-HSD and competitors for the binding domaine of the enzyme (32).

Corticosterone probably plays the greatest role in this context for the following reasons:

(i) Kinetic analysis of human kidney 11β-HSD (type II isoenzyme) revealed a higher affinity for corticosterone than for cortisol [K_m for corticosterone, approximately 10 nmol/l; for cortisol, 55 nmol/l] (10, 11)

(ii) After ACTH treatment of normal subjects (18, 19) and in patients with EAS (14, 24, 25), total plasma corticosterone levels are between 10^{-8} and 2×10^{-7} mol/l. The percentage transcortin binding of corticosterone is lower than that of cortisol [78% vs 90%] (27), leading to a higher unbound fraction of corticosterone in plasma compared with cortisol. We found an IC_{50} of 2×10^{-7} mol/l, thus demonstrating the physiologically relevant inhibiting effect of corticosterone on the renal conversion of cortisol to cortisone.

(iii) As the other steroids tested do not reach the plasms concentrations of corticosterone under ACTH overstimulation (18–26), their influence on renal 11β-oxidation seems to be less important.

(iv) Corticosterone itself binds with a slightly higher affinity than cortisol and aldosterone to recombinant human mineralocorticoid receptors (2). Just like plasma cortisol and plasma ACTH levels, plasma corticosterone levels in EAS are significantly higher than in pituitary Cushing's syndrome (14, 24, 25). Thus, a higher concentration of unmetabolized corticosterone acting as a potent mineralocorticoid may also contribute to the high incidence of hypokalemic alkalosis in patients with EAS.

18-OH-Corticosterone shows only a transient rise under ACTH excess (19, 20). Therefore, its inhibiting potency on 11β-HSD cannot play a pathophysiological role in EAS. It may contribute to the very fast effect of ACTH infusion on the cortisol/cortisone ratio (14, 15). As we are not aware of any data about the levels of 11-OH-androstenedione in the different forms of Cushing's syndrome (23, 26), a conceivable role of this ACTH-dependent steroid in EAS is only speculative. The contribution of DOC to the mineralocorticoid manifestation in EAS is discussed elsewhere (14, 26). Concerning this steroid, our study shows that DOC could exert an effect by binding to the mineralocorticoid receptor, but not by inhibiting 11β-HSD.

Walker et al. (14) assumed that 17-OH-progesterone and 17,20-OH-progesterone might act as ACTH-dependent inhibitors of 11β-HSD, because these steroids are essential for ACTH-induced hypertension in sheep (33). In our study, supraphysiological concentrations (18) of these steroids did not inhibit renal 11β-HSD.

Murphy (34) and Souness et al. (35) have shown that 11β-OH-progesterone and 11β-OH-pregnenolone are potent inhibitors of 11β-HSD. As plasma levels of these steroids in high ACTH states are not published, we cannot assess their role in EAS.

In human kidney slices, β-glycyrrhetinic acid shows an IC_{50} of approximately 10^{-6} mol/l on renal cortisol oxidation (Fig. 3). This is in keeping with the results of Monder et al. (36), who reported on an IC_{50} between 10^{-6} and 10^{-5} mol/l for glycyrrhetinic acid inhibition of 11β-HSD in intact rat kidney tubules. In microsomes and homogenates, the inhibiting potency of glycyrrhetinic acid is considerably greater: in microsomes and homogenates of rat kidneys, the IC_{50} is between 10^{-9} and 10^{-8} mol/l (36). In homogenates of CHOP cells transfected with human 11β-HSD II, Albiston et al. (11) found an IC_{50} of glycyrrhetinic acid between 10^{-8} and 10^{-7} mol/l. Nevertheless, our results with glycyrrhetinic acid prove the validity of our in vitro system for testing the inhibiting potency of steroidal compounds on 11β-HSD in tissue slices.

As a support of the theory of Ulick et al. (16), the conversion of cortisol to cortisone in renal kidney slices is concentration dependent over the whole range of cortisol concentrations tested (Fig. 4). We do not know whether this observation only reflects saturation of renal 11β-HSD. A rapid co-substrate consumption at higher substrate concentrations or a reduced transport capacity into the renal target cells are other mechanisms that may limit the percentage conversion of cortisol to cortisone in human kidney slices. Hellmann et al. (37) have reported that the affinity of [11-^{3}H]cortisol for 11β-HSD is less than that of the unlabeled cortisol because of its primary isotope effect. As we used [1,2,6,7-^{3}H]cortisol and have found similar results with unlabeled substrates (17), the possibility of a primary isotope effect is very unlikely.

Our model imitates the physiological in vivo conditions relatively well. In normal subjects the plasma concentration of unbound cortisol is between 10^{-8} and 6×10^{-8} mol/l (27). Ninety per cent of total plasma cortisol is tightly bound to transcortin, thus being prevented from entering the renal tubular cells and from acting as substrate for 11β-HSD. At physiological plasma conditions, transcortin is nearly saturated with cortisol. Supraphysiological cortisol levels, as in EAS, led to a disproportionate increase of unbound cortisol (27). In these circumstances the concentration of unbound cortisol in plasma and in the extracellular fluid can reach 10^{-6} mol/l. Thus, the cortisol concentrations chosen for experiment 3 (Fig. 4) comprise the whole pathophysiological range.

We cannot prove which type of 11β-HSD is responsible for the conversion of cortisol to cortisone in our slices prepared from human renal cortex. However, Whorwood et al. (38) have recently reported that 11β-HSD II mRNA was detected in human kidney cortex and medulla, whereas 11β-HSD I mRNA was only found in kidney medulla. In a previous paper (17), we found distinct differences between kidney cortex and liver slices with regard to the conversion of cortisone to cortisol: while 11-keto-reductase activity in the liver was high, it was almost absent in the kidney. These observations seem to indicate that our tissue preparations predominantly or exclusively test the type II isoenzyme in human kidneys.

In summary, we believe we have shown that the increased cortisol/cortisone ratio in plasma and urine under ACTH excess is mainly due to overload with endogenous substrates rather than to direct inhibition of renal 11β-HSD by ACTH or ACTH-dependent steroids. The important difference between an ACTH excess and isolated hypercortisolism is the ACTH-induced adrenal hypersecretion of other 11β-HSD substrates in addition to cortisol. Therefore, under ACTH-induced hypercortisolemia, more substrate molecules compete for the binding domains of 11β-HSD, thus leading to the higher cortisol/cortisone ratio during ACTH than during cortisol infusion (14, 15). Although our results may sufficiently explain the described in vivo observations (14–16), other regulatory effects of ACTH on 11β-HSD are possible. The known distribution of the ACTH receptor (30) makes an ACTH-induced extra-adrenal inhibitor of 11β-HSD unlikely. Other ACTH-dependent adrenal steroids or their metabolites not tested in our laboratory or an end-product inhibition of 11β-HSD (11) may play an additional role. Apart from effects on renal 11β-HSD, long-term elevations of glucocorticoids may induce 11β-HSD synthesis in different organs (6) and enhance the hepatic conversion of cortisone to cortisol (39).

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Received July 7th, 1995
Accepted October 16th, 1995

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0021-972X/99/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 84, No. 11
Printed in U.S.A.

Progesterone Metabolism in the Human Kidney and Inhibition of 11β-Hydroxysteroid Dehydrogenase Type 2 by Progesterone and Its Metabolites*

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ABSTRACT

Progesterone binds with high affinity to the mineralocorticoid (MC) receptor, but confers only very low agonistic MC activity. Therefore, progesterone is a potent MC antagonist *in vitro*.

Although progesterone reaches up to 100 times higher plasma levels in late pregnancy than aldosterone, the *in vivo* MC antagonistic effect of progesterone seems to be relatively weak. One explanation for this phenomenon could be local metabolism of progesterone in the human kidney, similar to the inactivation of cortisol to cortisone by the 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2. We studied the metabolism of progesterone in the human kidney *in vitro* and found reduction to 20α-dihydro (DH)-progesterone as the main metabolite. Ring-A reduction to 5α-DH-progesterone, 20α-DH-5α-DH-progesterone, and 3β,5α-tetrahydro (TH)-progesterone was also documented. We further showed for the first time that 17-hydroxylation of progesterone (17α-OH-progesterone, 17α-OH, 20α-DH-progesterone), normally localized in the adrenals and the gonads, occurs in the human adult kidney. We found no formation of deoxycorticosterone from progesterone in the human adult kidney. Using human kidney cortex microsomes, we tested the inhibitory potency of progesterone and its metabolites on the 11β-HSD type 2. The most potent inhibitor was progesterone itself ($IC_{50} = 4.8 \times 10^{-8}$ mol/L), followed by 5α-DH-progesterone ($IC_{50} = 2.4 \times 10^{-7}$ mol/L), 20α-DH-progesterone, 3β,5α-TH-progesterone, 17α-OH-progesterone, and 20α-DH-5α-DH-progesterone (IC_{50} between 7.7×10^{-7} mol/L and 1.3×10^{-6} mol/L). The least potent inhibitor was 17α-OH,20α-DH-progesterone. In addition to progesterone metabolism by the kidney, the inhibition of 11β-HSD type 2 by progesterone and its metabolites could be a second explanation for the weak MC-antagonist activity of progesterone *in vivo*. Inhibition of 11β-HSD type 2 leads to an increase of intracellular cortisol in a way that the local equilibrium between the MC agonist cortisol and the antagonist progesterone is shifted to the agonist side. (*J Clin Endocrinol Metab* **84:** 4165–4171, 1999)

RECENT studies have shown that progesterone binds to the human mineralocorticoid (MC) receptor with an affinity similar to or even higher than that of aldosterone and acts as a MC antagonist (1, 2). This explains the natriuretic potency of progesterone, first described 40 yr ago (3–5). During the luteal phase of the menstrual cycle and in pregnancy, plasma progesterone rises to about 15 and 500 nmol/L, respectively (6, 7), exceeding aldosterone concentration at least 20-fold (8). The mechanism by which aldosterone can act as a potent mineralocorticoid under these conditions is still an enigma.

The MC receptor binds aldosterone and cortisol (F) with equal affinity. Plasma-free F levels are ~50-fold higher than those of aldosterone. Funder *et al.* (9) and Edwards *et al.* (10) found that the renal 11β-hydroxysteroid dehydrogenase (11β-HSD) converts F into cortisone (E), which does not bind to the MC receptor, whereas aldosterone is not metabolized by 11β-HSD. Concerning F, the selectivity of the MC receptor for aldosterone is, therefore, enzyme-mediated and not receptor-specific.

Received April 27, 1999. Revision received July 22, 1999. Accepted August 8, 1999.

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* Supported by Grant DI 741/1–1 from Deutsche Forschungsgemeinschaft (DFG). This work is dedicated to Prof. Klaus Hierholzer, M.D., a pioneer of renal endocrinology, on the occasion of his 70th birthday.

In analogy to these findings, we hypothesize that progesterone may be metabolized by enzymes of MC receptor target tissues. We, therefore, studied the progesterone metabolism in human renal cortical and medullary homogenates, as well as in subcellular fractions of human kidneys.

In addition, progesterone and its renal metabolites could influence 11β-HSD type 2 (11–13). By inhibiting 11β-HSD type 2, F could gain more access to the MC receptor, thus offsetting part of the anti-MC effect of progesterone. Therefore, progesterone and its metabolites were tested for their inhibitory potency on 11β-HSD type 2 in microsomes of human renal cortex.

Materials and Methods

Materials

4-C^{14}-progesterone (0,02 mCi/mL) and [1,2,6,7-^{3}H(n)]-F (70.0 Ci/mmol) were obtained from NEN Life Science Products; unlabelled progesterone (4-pregnene-3, 20-dione) was from Serva Feinbiochemica (Heidelberg, Germany). 4-pregnen-17α-ol-3, 20-dione (17α-OH-progesterone) and 4-pregnene-21-ol-3, 20-dione [deoxycorticosterone (DOC)] were purchased from Makor Chemicals Ltd. (Jerusalem, Israel); and 17α-OH,20α-DH-progesterone (4-pregnen-17, 20α-diol-3-one) was from Paesel and Lorei GmbH and Co. (Frankfurt/M, Germany).

The following steroids and substances were obtained from Sigma Chemical Co. (St. Louis, MO): F, E, androstenedione (4-androsten-3, 17-dione), 20α-DH-progesterone (4-pregnen-20α-ol-3-one), 20β-DH-progesterone (4-pregnen-20β-ol-3-one), 20α-DH-3β,5α-TH-progesterone (5α-pregnane-3β, 20α-diol), 20α-DH-3α,5β-TH-progesterone (5β-pregnane-3α, 20α-diol), 20α-DH-3α,5α-TH-progesterone (5α-pregnane-3α, 20α-diol), 20α-DH-5α-DH-progesterone (5α-pregnane-20α-ol-3-

one), 5β-DH-progesterone (5β-pregnane-3, 20-dione), 3β,5α-TH-progesterone (5α-pregnane-3β-ol-20-one), 5α-DH-progesterone [5α-pregnane-3, 20-dione (allo)], D-glucose-6-phosphate monosodium salt, sucrose, β-NAD^+ (nicotinamid-adenin-dinucleotid), β-NADPH-tetrasodium-salt, and β-NADH-disodium-salt.

The following steroids were purchased from Steraloids Inc. (Wilton, NH): 3α,5α-TH-progesterone (5α-pregnane-3α-ol-20-one), 3α,5β-TH-progesterone (5β-pregnane-3α-ol-20-one), 20α-DH-3β,5β-TH-progesterone (5β-pregnan-3β, 20α-diol), 6β-OH-progesterone (4-pregnen-6β-ol-3, 20-dione), 6α-OH-progesterone (4-pregnen-6α-ol-3, 20-dione), 3β,5β-TH-progesterone (5β-pregnan-3β-ol-20-one), and 6α-OH-3α,5β-TH-progesterone (5β-pregnan-3α, 6α-diol-20-one).

Glucose-6-phosphate-dehydrogenase (1000 U/mL) from Leuconostoc mesenteroides was obtained from Boehringer Mannheim (Mannheim, Germany); carbenoxolone and 18β-glycyrrhetinic acid was from Aldrich-Chemie (Steinheim, Germany); ethylendichloride, n-hexane, 2-propanol, 1-hexanol, methylacetate, 1,2-dichlorethane, 97% sulfuric acid, acetic anhydride, sodiumhydrogen-phosphate-dihydrate, and sodiumhydrogen-phosphate-monohydrat were from Merck Ltd. (Darmstadt, Germany); and methanol in LiChrosolv-quality was from J.T. Baker B.V. (Deventer, the Netherlands).

Human kidney tissue, in the urology department of our hospital, was taken from unaffected kidney segments that were removed because of renal cell carcinoma (14).

Methods

Metabolism experiments

Preparation and incubation of homogenates. We used kidneys of two males (56 and 58 yr of age) and two postmenopausal women (52 and 67 yr of age). Cortex and medulla were separated macroscopically. The tissue of each patient was cut immediately into small pieces and homogenized separately (Ultra-Turrax TP18 from Janke and Kunkel GmbH, Staufen, Germany; Potter S from B. Braun) with sodium-phospate buffer (0.01 mol/L, pH 7.0) and 0.25 mol/L sucrose. All steps of preparation were performed on ice. Renal cortex (n = 3 for each patient) and medulla (n = 3 for each patient) were incubated without or with cosubstrate (10^{-3} mol/L NADH or NADPH) and a NADH/NADPH-regenerating enzyme system (containing 10^{-2} mol/L glucose-6-phosphate and 10 U glucose-6-phosphate-dehydrogenase). Total incubation volume was 1 mL (pH 7.0) containing 150 mg homogenized tissue, 200.000 cpm 4-C^{14}-progesterone (10^{-9} mol/l) dissolved in 10 μl methanol, cosubstrate and regenerating system or blanks. Incubation time was 120 min in a 37 C heated shaking water bath. The incubation was stopped by placing the incubating set on ice and adding 1 mL methylacetate to each well.

Preparation and incubation of subcellular fractions: Renal cortex and medulla from eight male patients (age 51–75 yr of age) were cut into small pieces and homogenized as described before. All subsequent steps were performed at 0–4 C using the method of Lakshmi and Monder (15). The homogenates were centrifuged at 750 × *g* for 30 min. The pellet was disposed, and the supernatant was centrifuged at 20,000 × *g* for 30 min. The pellet containing the mitochondria was stored in liquid nitrogen. The supernatant was centrifuged at 105,000 × *g* for 60 min. The generated new supernatant was frozen as cytosolic fraction. The pellet was resuspended in the homogenizing buffer and centrifuged again at 105,000 × *g* for 60 min. The washed microsomal pellets were resuspended in sodium-phosphate buffer and stored in liquid nitrogen. The subcellular fractions were used within 10 months, and no loss in enzyme activity was seen during this period. Protein was quantified before every incubation using Bradford analysis (Bio-Rad Laboratories, GmbH, München, Germany).

After preliminary studies for time kinetics, protein kinetics, and cosubstrate preferences (data not shown), we determined the following conditions for all subcellular incubations (each with n = 5): the incubation time was 2 h in a shaking water bath (37 C); the total incubation volume was 1 mL (pH 7.0), the cosubstrate concentration (NADPH) was always 10^{-3} mol/L, the amount of protein in each well was 800 μg, and all incubations included the coenzyme-regenerating system described above. Additional incubations (n = 3) were performed with increasing concentrations (10^{-8} to 10^{-6} mol/L) of unlabelled progesterone in the cytosolic and microsomal fraction of kidney cortex. The incubation was stopped by placing the incubation system on ice and by adding 1 mL methylacetate to each well.

Extraction and detection of progesterone and its metabolites. For extraction of the steroids and detection by two-dimensional thin-layer chromatography (TLC), we modified the methods described by Blom *et al.* (16) and Nienstedt (17, 18).

Each well was centrifuged at 1000 × *g* and 4 C for 20 min. The upper phase containing methylacetate and the steroids was pipetted into a separate tube. To the remaining lower phase, 700 μL methylacetate were added, vortexed, and centrifuged again. The upper phase was separated, and the lower phase was resuspended. The washing procedure was repeated four times. The radioactivity of the remaining lower phase containing buffer and possible water soluble-conjugated steroids was checked in a β-counter (Minaxi Tri-Carb 4000 Series; Packard Instruments B.V., Groningen, the Netherlands) and was always less than five percent. The collected upper phases (free steroid fractions) were evaporated and stored at −20 C until detection.

TLC. The free steroid fractions were redissolved in methanol and pipetted onto TLC plates (TLC-alumina plates 20 × 20 cm, coated with silicagel 60_{F254}; Merck Ltd., Darmstadt, Germany). A methanol solution (10 μL) with 20 unlabelled authentic reference steroids (containing 10 μg of each steroid; see materials listed above) was pipetted on the TLC plate starting point, as well. One TLC plate was used for each sample. The plates were run two-dimensionally using a mixture of methylacetate-ethylendichloride (65:35) for one direction (50 min), and hexanol-hexane (75:25) for the other direction (210 min).

After drying the plates at 150 C for 30 min, they were scanned for radioactivity with a Berthold Linear Analyzer LB284/LB285 Chroma 2D (with 90% argon and 10% methan gas; Berthold GmbH and Co., Wildbad, Germany), and a recovery of ~63% was calculated. No corrections for estimated average losses were made. The total sum of radioactivity (progesterone and metabolites) found was defined as 100%, and the percentage of the newly formed metabolites are being reported. For identification of the newly formed metabolites, the added unlabelled reference steroids were stained with Liebermann-Burchard reagent (ethanol-acetic anhydride-sulfuric acid), heated at 115 C for 30 min, and located under UV light (360 nm) (Fig. 1). Due to the R_F-values (ratio of velocity of the solute relative to the velocity of the solvent front) and the R_M-values ($\log_{10}$ ($1/R_F$ -1) (17), the radioactive metabolites were identified.

Because of no clear separation of 20α-DH-progesterone and 20β-DH-progesterone by TLC, we used a normal-phase high-performance liquid chromatography (HPLC) for these steroids. An aliquot of each free steroid fraction and a mixture of unlabelled reference steroids (progesterone, androstenedione, 20α-DH-progesterone, and 20β-DH-progesterone) were injected into the HPLC column that was run with an isocratic solvent mixture (95% hexane and 5% 2-propanol, flow-rate 1.3 mL/min) for 20 min. The steroids were located on the chromatogram by UV-detection (240 nm) and by C^{14}-detection using a radioactivity monitor, as described previously (14). The progesterone metabolite, located on the 20β-DH-/20α-DH-progesterone spot in the TLC, showed the retention time of 20α-DH-progesterone, and not as 20β-DH-progesterone, in the HPLC chromatogram.

11β-HSD type 2 experiments. Microsomes were prepared from kidney cortex of six different kidneys (three male and three female patients; age, 47–81 yr; mean, 60 yr), as described previously (19). Protein quantification was done by Bradford analysis. The incubation conditions were constant: the total incubation volume was 1 mL, the cosubstrate (NAD^+) was 1 mmol/L, 100.000 cpm ^{3}H-F (2 nmol/l) as tracer and 25 nmol/l unlabelled F, using a 0.1 mol/L sodium phosphate buffer (pH 8.0), and progesterone and its metabolites (5α-DH-progesterone, 20α-DH-progesterone, 17α-OH-progesterone, 17α-OH, 20α-DH-progesterone, 20α-DH-5α-DH-progesterone, and 3β, 5α-TH-progesterone) in concentrations ranging from 10^{-9} to 10^{-5} mol/L as inhibitors. Carbenoxolone and glycyrrhetinic acid were used as reference inhibitor substances. The incubation time was 30 min. The incubation was started by the addition of microsomes (0.03 mg/mL) to 10-min preincubated wells containing all assay components, except the enzyme preparation. In all assays, blanks were included containing all assay components, except buffer, instead of the enzyme preparation. Incubations were terminated by the

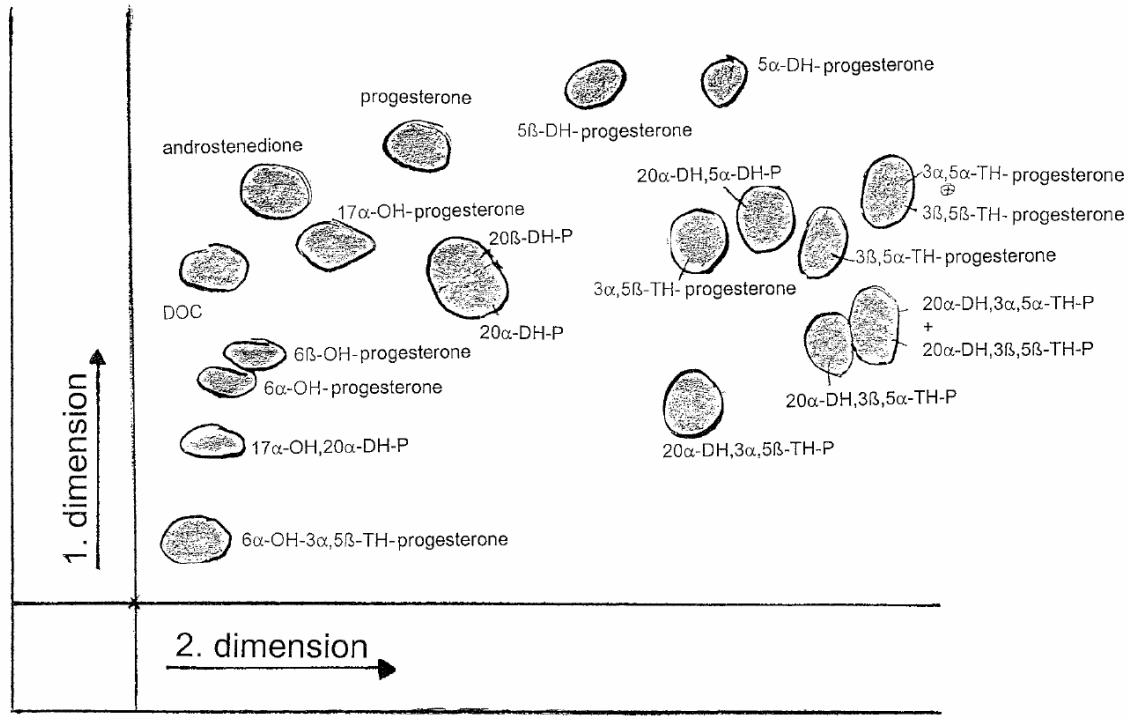

FIG. 1. Separation of progesterone (P), androstenedione, DOC, and 18 progesterone metabolites with two-dimensional TLC. The first dimension was run with methylacetate:ethylendichloride (65:35); second dimension with hexanol:hexane (75:25). TLC plates were stained with Liebermann-Burchard reagent and heated afterward. The reference steroids were located under UV light (360 nm).

addition of 2 mL cold methanol and by transfer on ice. The steroids were extracted by Sep-Pak C_{18}-cartridges (Waters Millipore Corp. GmbH, Eschborn, Germany). The separation of F and E was performed by TLC, as described previously (19). The steroid spots were identified under UV light, cut out, and transferred to scintillation vials. The cut out pieces containing F or E were incubated in a scintillation liquid for 12 h. The amounts of F and E were measured in a β-counter so that the percentage conversion could be calculated.

Statistics: Student's t test was used for statistical calculations comparing the amount of each progesterone metabolite formed in cortical *vs.* medullary cytosol and in cortical *vs.* medullary microsomes (Table 1). Duncan's multiple range test was used for multiple comparisons of progesterone metabolites formed in the presence of increasing amounts of unlabelled progesterone (Fig. 3).

Results

Metabolism experiments

Homogenates. In renal homogenates, no conjugated progesterone metabolites were found, and the progesterone metabolism was low without the NADH/NADPH-regenerating system (data not shown). NADPH was the preferred cosubstrate for progesterone metabolism in renal homogenates. The main renal progesterone metabolite found in both sexes was 20α-DH-progesterone (Fig. 2). We also found 17α-OH-progesterone and 17α-OH,20α-DH-progesterone. Ring-A reduction was also detectable, leading to the formation of 5α-DH-progesterone, 20α-DH-5α-DH-progesterone, and 3β,5α-TH-progesterone. We found no formation of DOC from progesterone in adult kidney homogenates. Metabolism in renal cortex was of greater diversity and extent than in renal medulla. Female and male kidney homogenates formed the same progesterone metabolites, but the intensity of metabolism seems to be greater in female kidney homogenates.

TABLE 1. Metabolites formed from ^{14}C-progesterone (10^{-9} mol/L; in percent conversion) in cytosol and microsomes of male human kidney cortex and medulla. Conditions of incubation: cosubstrate NADPH (10^{-3} mol/L), NADH/NADPH-regenerating system, 800 μg protein, 120-min incubation time (means ± SD).

Substance	Cortex cytosol (n = 5)	Medulla cytosol (n = 5)
20α-DH-P[a]	27.1 ± 1.2%	26.3 ± 1.0%
Substance	**Cortex microsomes (n = 5)**	**Medulla microsomes (n = 5)**
17α-OH-P	31.4 ± 0.8%	22.2 ± 2.0%[b]
17α-OH,20α-DH-P	11.7 ± 1.3%	7.1 ± 1.1%[b]
5α-DH-P	6.6 ± 0.9%	1.9 ± 0.7%[b]
3β,5α-TH-P	0.4 ± 0.4%	0.3 ± 0.7%
20α-DH-P	0.9 ± 0.3%	1.8 ± 0.4%

[a] P = progesterone.
[b] $P < 0.001$ compared to cortex.

Subcellular fractions. In incubations of cytosol and microsomes, no conjugated progesterone metabolites were found. NADPH was the preferred cosubstrate (data not shown). In the cytosolic fraction of kidney cortex and medulla, 20α-DH-progesterone was the only metabolite formed (Table 1).

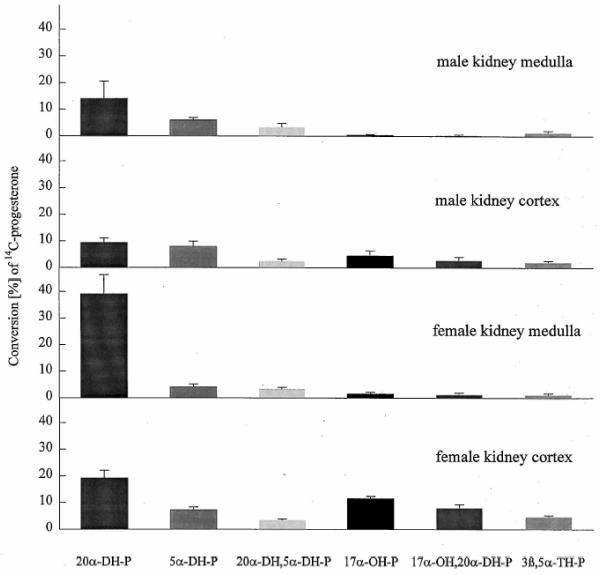

FIG. 2. ^{14}C-progesterone metabolism (percentage conversion) in medulla and cortex kidney homogenates of two male and two female patients with NADPH and a NADH/NADPH-regenerating system. Means ± SE of triplicates from each kidney. Data not shown for metabolism with NADH as cosubstrate, which showed the same metabolites but less turnover. P = progesterone.

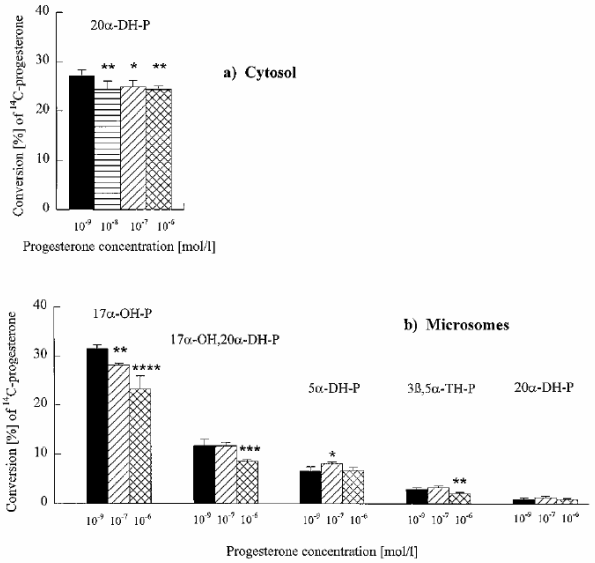

FIG. 3. ^{14}C-progesterone (P) metabolism (percentage conversion) in cytosol (a) and microsomes (b) of male human kidney cortex with ^{14}C-progesterone (10^{-9} mol/L) and increasing concentrations of unlabelled progesterone (10^{-8}–10^{-6} mol/L). Cosubstrate: NADPH (10^{-3} mol/l). A NADH/NADPH-regenerating system was used, 800 μg protein was used, 120 min incubation time. Means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.001$ compared with 10^{-9} mol/L progesterone.

There was no significant difference in progesterone metabolism between cytosol from renal cortex and medulla. The cortical and medullary microsomes showed a greater variability of progesterone metabolites (17α-OH-progesterone, 17α-OH, 20α-DH-progesterone, and 5α-DH-progesterone) than cytosol, but only traces of 3β,5α-TH-progesterone and 20α-DH-progesterone were found. In microsomes of the cortex, over 50% of progesterone was metabolized, whereas the medullary microsomes were less active.

On adding increasing unlabelled progesterone concentrations to kidney cortex cytosol, the conversion of ^{14}C-progesterone to 20α-DH-progesterone decreased slightly, but significantly ($P < 0.01$) (Fig. 3). In cortex microsomes, the conversion to its metabolites, especially to 17α-hydroxylated metabolites, was reduced to a greater extent ($P < 0.001$) at the highest concentration of added unlabelled progesterone tested (10^{-6} mol/L).

11β-HSD type 2 experiments

Carbenoxolone and glycyrrhetinic acid are known to be potent inhibitors of 11β-HSD type 2. Similar to published results, we found IC_{50}'s in the nanomolar range (Table 2). Progesterone and all its renal metabolites also inhibited the conversion of F to E by 11β-HSD type 2 in human renal cortex microsomes at F concentrations of 25 nmol/L. Of these steroids, the most potent inhibitors were progesterone itself (IC_{50} = 48 nmol/L) and 5α-DH-progesterone (IC_{50} = 240 nmol/L) (Table 2), followed by 20α-DH-progesterone, 3β,5α-TH-progesterone, 17α-OH-progesterone, 20α-DH-5α-DH-progesterone with similar IC_{50} between 0.77 and 1.3 μmol/L. The least potent inhibitor was 17α-OH,20α-DH-progesterone, for which no IC_{50} could be calculated in the range of concentrations tested.

TABLE 2. Inhibition of 11β-HSD type 2 in human renal cortex microsomes by glycyrrhetinic acid, carbenoxolone, progesterone, and its renal metabolites.

Substance	IC_{50}[a] [mol/L]
Glycyrrhetinic acid	8.0×10^{-9}
Carbenoxolone	8.4×10^{-9}
Progesterone	4.8×10^{-8}
5α-DH-progesterone	2.4×10^{-7}
20α-DH-progesterone	7.7×10^{-7}
3β,5α-TH-progesterone	8.0×10^{-7}
17α-OH-progesterone	9.0×10^{-7}
20α-DH-5α-DH-progesterone	1.3×10^{-6}
17α-OH,20α-DH-progesterone	$>10^{-5}$

[a] IC_{50}, concentration of half-maximal inhibition of 11β-oxidation of F to E. Cosubstrate NAD^+ (10^{-3} mol/l), 100,000 cpm ^{3}H-cortisol as tracer, 25 nmol/L unlabelled cortisol, incubation time 30 min, 0.03 mg/mL protein.

Discussion

During the luteal phase of the menstrual cycle, progesterone plasma concentrations range between 30–110 nmol/L. During pregnancy the concentrations rise steadily until they peak at the end of the 3rd trimester in the range of 320–700 nmol/L (6, 7). In contrast, plasma aldosterone increases only slightly during the luteal phase and late pregnancy (0.6 nmol/L and 5.8 nmol/L, respectively) (8). Regarding the high-binding affinity of progesterone to the MC receptor (1, 2), it is still a question how aldosterone can keep

its function as effective MC agonist in the presence of high concentrations of progesterone. Several explanations have been put forward. First, one should take into account that only ~3% of progesterone is unbound, whereas 80% is weakly bound to albumin and 17% to corticosteroid-binding globulin. On the other hand 37% of aldosterone is unbound and thereby available for intracellular action (20). Second, the half-life of the aldosterone-MC receptor complex was estimated to be about 600 min compared with 45 min of the progesterone-MC receptor complex (21). A third mechanism that possibly keeps the MC receptor clear of the antagonist progesterone is the extra-adrenal formation of DOC, a weak MC, from plasma progesterone. Some authors (22–25) reported DOC biosynthesis in human fetal kidney tissue, which implies the idea of para- or autocrine formation of a MC receptor agonist in its site of action. Winkel *et al.* (22) found in kidney microsomes of a pregnant woman, a 65-yr-old woman, and two 56- and 31-yr-old men less than 1% conversion of progesterone to DOC, but they failed to show any activity in 10 other kidneys. In our experiments, we found no formation of DOC from progesterone in adult kidneys. Because the conversion rates of progesterone to DOC reported by Winkel *et al.* (22) were markedly less than 1%, it is possible that our detection system was too insensitive to measure such low conversion rates. It is also likely that the conversion of plasma progesterone to DOC varies widely among individuals, suggesting genetic differences in the capacity of extra-adrenal 21-hydroxylation. On the other hand, it is possible that particular hormonal circumstances, like pregnancy or fetal life, are linked with the capacity of renal conversion of progesterone to DOC.

Progesterone metabolism

We suggest that the MC receptor is partly protected from high progesterone concentrations during pregnancy by its metabolic inactivation similar to the inactivation of F to E by 11β-HSD type 2. There are only a few studies on progesterone metabolism in extrahepatic and extragonadal human tissues. Nienstedt *et al.* (26) described progesterone metabolism in the human adult small intestine with 3β,5α-TH-progesterone, 3α,5α-TH-progesterone, 20α-DH-5α-DH-progesterone, and 20α-DH-progesterone as the main metabolites. In the human fetal intestine, metabolism was shifted toward the 5β-metabolites. Blom *et al.* (16) examined progesterone metabolism in human parotid and submandibular glands, and Ojanotko-Harri (27) in the human gingiva. Both groups found 20α-DH-progesterone and 5α-DH-progesterone as the main metabolites. Until now, progesterone metabolism was described only in the human fetal kidney with formation of 20α-DH-progesterone, 3α,5β-TH-progesterone, 20α-DH-3α,5β-TH-progesterone, and DOC (28).

Our experiments show that progesterone is converted in considerable quantity into various metabolites in human adult renal tissue with 20α-DH-progesterone as the main metabolite. These results are similar to the metabolite profile described in human parotid and submandibular glands (16). The preferred cosubstrate with kidney cortex microsomes was NADPH, and the rate of progesterone inactivation/metabolism amounted to ~50% in 2 h. The 20α-HSD seems to be localized mainly in the cytosol. Recently, Soucy *et al.* (29) described the isolation of human 20α-HSD cDNA , but until now no studies are available about human 20α-HSD mRNA expression in human tissues. The other metabolites found suggest the presence of 5α-reductase, 3β-hydroxysteroid dehydrogenase and, surprisingly, 17-hydroxylase. To our knowledge, this is the first description of the formation of 17α-OH-progesterone from progesterone in the human adult kidney, which is believed to occur in the adrenals and gonads only.

Whether the progesterone metabolites found bind to the human MC receptor and have an antagonist or agonist effect remains to be tested. So far, only two groups examined the competition between ^{3}H-aldosterone and 17α-OH-progesterone and 17α-OH,20α-DH-progesterone for the ovine and rat MC receptor (30, 31) and found no significant binding of these metabolites.

Incubations with increasing concentrations of unlabelled progesterone (up to 10^{-6} mol/L) in human renal cortex cytosol (Fig. 3a) showed only a small (27.1% to 24.3%), but significant ($P < 0.01$), decrease in percentage conversion due to saturation in metabolic activity, suggesting a very effective and potent enzyme system (20α-HSD). The percentage of 17-hydroxylation in human renal cortex microsomes (Fig. 3b) is reduced to a greater extend in the presence of 10^{-6} mol/L progesterone (17α-OH-progesterone: 31.4% to 23.2%; 17α-OH, 20α-DH-progesterone: 11.7% to 8.6%). However, more than 40% of progesterone are metabolized altogether at this high concentration. Because there was only a small increase in the percentage conversion of progesterone into metabolites with incubation times longer than 60 min (data not shown), the metabolism of progesterone in the kidney seems to occur relatively fast.

Inhibition of 11β-HSD type 2

Progesterone is a known potent inhibitor of 11β-HSD type 2, with IC_{50} ranging from 6 nmol/L (11) up to 1.1 μmol/L, depending on protein origin and assay (12, 13). There are only few studies in the literature on inhibition of 11β-HSD type 2 by progesterone metabolites (11, 12). We found that the renal 11β-HSD type 2 of kidney cortex microsomes is inhibited by progesterone itself and by the renal progesterone metabolites detected (Table 2). We demonstrated an inhibition of human renal 11β-HSD type 2 by 3β,5α-TH-progesterone and 20α-DH-5α-DH-progesterone for the first time. Progesterone probably plays the main part in inhibiting 11β-HSD type 2 due to its low IC_{50}-value and its high plasma concentration. At least during the 3rd trimester of pregnancy, it is likely that free and albumin-bound progesterone concentrations are exceeding 50 nmol/L, the concentration of half maximal inhibition of 11β-HSD type 2. Whether 5α-DH-progesterone, the second potent inhibitor, and the other metabolites have a significant inhibitory influence *in vivo* is speculative because no information on intracellular and only few information on plasma concentrations (20α-DH-progesterone: up to 90 nmol/L in 3rd trimester, 17α-OH-progesterone: up to 29 nmol/L in 3rd trimester (32) are available yet.

The inhibition of 11β-HSD type 2 by progesterone and its

metabolites could lead to a decreased inactivation of F to E by 11β-HSD type 2, and, therefore, to an increase of intracellular F concentration. This could result in a greater access of F to the MC receptor and, consequently, in a further decrease of the anti-MC effect of progesterone on the MC receptor (Fig. 4).

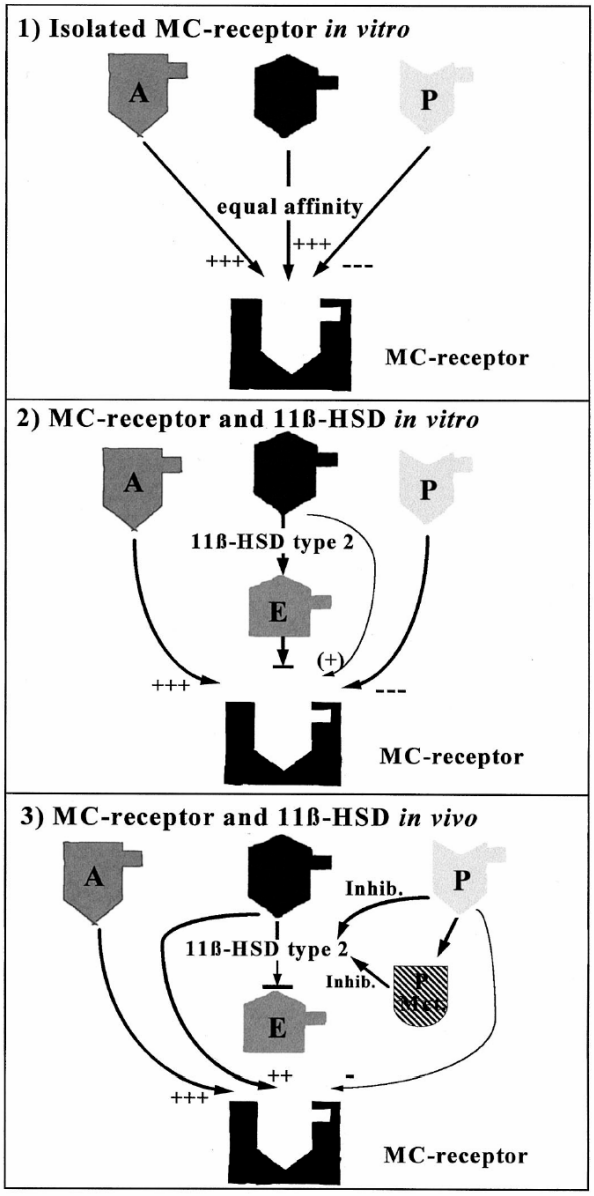

FIG. 4. Hypothesis of progesterone (P)/MC interaction. 1, Aldosterone (A), F, and P bind with similar affinity to the MC receptor: A and F activate (+++); P blocks (—) the MC receptor. 2, Renal 11β-HSD type 2 converts F to E that does not bind to the MC receptor. 3, Metabolism of P diminishes the MC receptor blockade by P, but P and its renal metabolites inhibit 11β-HSD type 2, thus increasing activation of the MC receptor by F. Inhib. = inhibition, Met. = metabolites.

Clinical implications

During normal pregnancy, the renin-angiotensin-aldosterone system is activated (33), possibly as a partial compensation for the anti-MC effect of high progesterone levels. Preeclampsia (defined as hypertension), edema, and proteinuria in the 3rd trimester, is associated with exaggerated sodium retention. One mechanism of sodium retention could be an increased activation of the MC receptor, supported by the observation of a decreased MC receptor count of mononuclear leukocytes in the state of preeclampsia (34). This receptor down-regulation, similar to states of MC excess, contrasts with the reduced serum aldosterone observed in patients with preeclampsia compared to normal pregnancies (33, 35, 36). No abnormalities in F (37) or DOC (38) concentrations were reported in preeclampsia. Some authors found increased progesterone production in preeclamptic placentas (39, 40), but others describe an unchanged plasma progesterone concentration in preeclampsia compared to normal pregnancy (35, 36, 41).

Inhibition of 11β-HSD type 2 by progesterone and its metabolites, as described in this article, and a subsequently increased intracellular F (MC agonist) concentration, could be one explanation for MC receptor down-regulation (34) and the decreased PRA and serum aldosterone (33, 35, 36) in preeclampsia. An altered progesterone metabolism in the kidney and an inhibited 11β-HSD type 2 could, therefore, participate in the pathogenesis of preeclampsia. Walker *et al.* (42) measured the free F/E ratio in urine of preeclamptic women and found no difference to normal pregnancy, but only a very small number of patients was examined in this report. Very recently, Heilmann *et al.* (43) confirmed the hypothesis of inhibited 11β-HSD type 2 in preeclampsia by showing an increased free F/E ratio in the urine of 41 patients with preeclampsia compared with 48 women with normal pregnancy.

In conclusion, we found a marked metabolic activity toward progesterone in human adult kidneys, which could be one mechanism of reducing the MC antagonistic influence of progesterone similar to inactivation of F by 11β-HSD type 2. Moreover, progesterone and its metabolites are potent inhibitors of 11β-HSD type 2 and could hereby increase the intracellular F concentration, producing more MC agonist activity.

We are planning to localize the progesterone metabolizing enzymes in the kidney. In this regard, it will be of special interest to find out whether some of these enzymes are colocalized with the MC receptor and the 11β-HSD type 2 in the distal tubule and the collecting duct.

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Erratum

A correction has been made in the article "Lack of Sex Difference in Cerebrospinal Fluid (CSF) Leptin Levels and Contribution of CSF/Plasma Ratios to Variations in Body Mass Index in Children" by Annett Wiedenhöft *et al.* (*The Journal of Clinical Endocrinology & Metabolism* **84:**3021–3024). On page 3023, the second to last paragraph of the article, which cited Refs. 22 and 23, has been deleted. The references were not included in the reference list. The authors regret the error.

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Molecular and Cellular Endocrinology 171 (2001) 21–24

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Enzyme-mediated protection of the mineralocorticoid receptor against progesterone in the human kidney

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Abstract

Progesterone (P) is a mineralocorticoid (MC)-antagonist in vitro. During pregnancy, plasma P concentrations exceed aldosterone concentrations at least 50-fold, but plasma aldosterone increases only 4–8-fold in a compensatory manner. Since the in vivo anti-MC activity of P seems to be only moderate, we hypothesized that P is metabolized by enzymes of MC target tissue similar to the way cortisol is metabolized by 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2 in order to protect the MC receptor. We, therefore, examined P metabolism using 4-^{14}C-P in subcellular fractions of human postmenopausal and male kidneys, and in homogenates of one premenopausal kidney. We found that P is converted effectively, even at high P concentrations (10^{-6} mol/l), to various metabolites: 20α-dihydro(DH)-P; 17α-OH-P; 17α-OH,20α-DH-P; 5α-DH-P; 3β,5α-tetrahydro(TH)-P; and 20α-DH,5α-DH-P. Homogenates of premenopausal kidney also showed conversion to 3α- and 5β-reduced P metabolites. These results confirm the existence of an efficient renal enzyme system as a possible mechanism of an enzyme-mediated MC receptor selectivity.

Keywords: Progesterone; Mineralocorticoid receptor; Progesterone metabolism

1. Introduction

The mineralocorticoid (MC) receptor exhibits in vitro the same affinity to the mineralocorticoid aldosterone, the glucocorticoid cortisol (F), and the gestagen progesterone (Rupprecht et al., 1993; Myles and Funder, 1996). In MC receptor target tissues, such as the kidney, the colon, and the salivary glands, the unselective MC receptor is protected by an enzyme, which belongs to the family of short chain alcohol dehydrogenases, named 11β-hydroxysteroid dehydrogenase (11β-HSD) type 2. This enzyme confers the MC receptor specificity in vivo towards aldosterone by converting F to its inactive 11-oxo metabolite cortisone (E) (Edwards et al., 1988; Funder et al., 1988). Progesterone (P) binds in vitro with similar affinity to the MC receptor as aldosterone, but confers only low agonistic MC activity. Therefore, P is an MC-antagonist in vitro.

During the luteal phase of the menstrual cycle, plasma P concentrations range between 30 and 110 nmol/l. During pregnancy, P concentrations rise steadily up to 320–700 nmol/l at the end of the third trimester. In contrast, aldosterone increases only slightly during the luteal phase and in late pregnancy (0.6 and 5.8 nmol/l, respectively). Considering the high affinity of P to the MC receptor and the fact that P concentrations exceed aldosterone concentrations at least 30- and 50-fold, it is still unclear how aldosterone can act as an MC under these conditions. We hypothesized that P is metabolized by enzymes of MC target tissue similar to the way F is inactivated by renal 11β-HSD type 2. Therefore, we examined P metabolism in the human kidney.

2. Methods

Microsomes, cytosol, mitochondrial, and nuclear fractions were prepared from the medulla and cortex of nine postmenopausal kidneys and eight male kidneys using the method of Lakshmi and Monder (1988). The subcellular fractions (800 μg protein for each incubation) were incubated ($n = 5$) for 120 min. with 4-^{14}C-P (10^{-9} mol/l, 200 000 cpm), cosubstrate NADPH (10^{-3}

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0303-7207/01/$ - see front matter
PII: S0303-7207(00)00429-9

mol/l), and a NADH/NADPH-regenerating system (containing 10^{-2} mol/l glucose-6-phosphate and 10 U glucose-6-phosphate-dehydrogenase) at pH 7.0. Additional incubations ($n = 3$) were performed with increasing concentrations (10^{-8}–10^{-6} mol/l) of unlabelled progesterone in the cytosolic and microsomal fraction of male kidney cortex. We used one premenopausal kidney for preparation of homogenates using the same method (Lakshmi and Monder, 1988). Homogenates were incubated the same way that the subcellular fractions were except that we used 300 mg of homogenized tissue for each incubation ($n = 5$). For identification of P-metabolites, each probe was extracted with methylacetate and run with two-dimensional thin-layer chromatography (TLC), using methylacetate-ethylendichloride (65:35) for one direction (50 min), and hexanol-hexane (75:25) for the other direction (210 min). The 4-^{14}C-labelled P and its metabolites were detected by a TLC Berthold Linear Analyzer LB284/LB285 Chroma 2D scanner (Fig. 1) and identified with unlabelled reference steroids, which were pipetted on the TLC starting point as well. In addition, the incubated probes were run with normal-phase high-performance liquid chromatography (HPLC) (Quinkler et al., 1999).

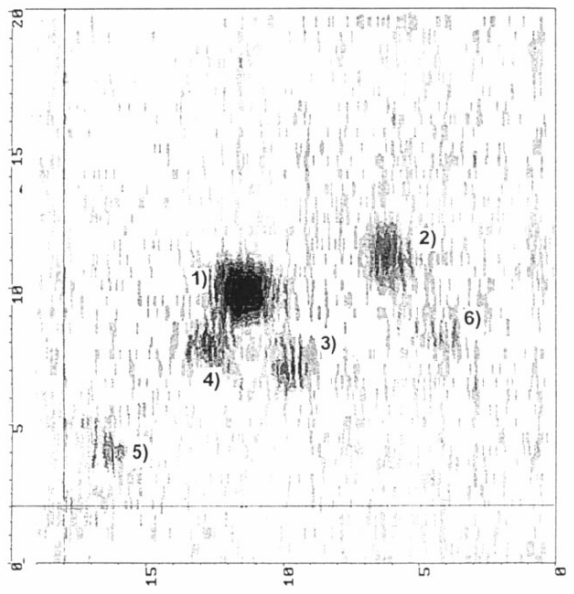

Fig. 1. Metabolism of 4-^{14}C-progesterone in renal cortex homogenates of a postmenopausal woman. Separation with two-dimensional thin-layer chromatography (TLC) and detection with a TLC scanner. (1) Progesterone; (2) 5α-dihydro-progesterone; (3) 20α-dihydro-progesterone; (4) 17α-hydroxy-progesterone; (5) 17α-hydroxy,20α-dihydro-progesterone; (6) 3β,5α-tetrahydro-progesterone.

Table 1
Percent conversion of ^{14}C-progesterone (10^{-9} mol/l) in cytosolic and microsomal fraction of human male kidney cortex ($n = 3$) and increasing concentrations (10^{-8}–10^{-6} mol/l) of unlabelled progesterone (P)[a]

Cytosol	10^{-9} mol/l P	10^{-8} mol/l P	10^{-7} mol/l P	10^{-6} mol/l P
20α-DH-P	26.8 ± 1.0	24.2 ± 0.7**	24.8 ± 1.3*	24.3 ± 1.7**
Microsomes	10^{-9} mol/l P	10^{-8} mol/l P	10^{-7} mol/l P	10^{-6} mol/l P
17α-OH-P	31.5 ± 0.9	30.3 ± 1.7	28.1 ± 0.4**	23.2 ± 2.7****
17α-OH, 20α-DH-P	11.6 ± 1.4	12.4 ± 1.0	11.7 ± 0.7	8.5 ± 0.3***
5α-DH-P	6.6 ± 1.0	6.9 ± 0.7	8.1 ± 0.4*	6.8 ± 0.5
3β,5α-DH-P	3.0 ± 0.4	2.7 ± 0.4	3.3 ± 0.3	2.0 ± 0.3**
20α-DH-P	1.0 ± 0.3	1.8 ± 0.0****	1.3 ± 0.3	0.9 ± 0.3

[a] NADPH 10^{-3} mol/l, 800 μg protein, 120 min incubation time. Means ± S.D. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, ****$P < 0.001$ compared with 10^{-9} mol/l P. DH, dihydro, TH, tetrahydro.

3. Results

Only 20α-dihydro(DH)-P was found in the cytosol fraction in renal cortex and medulla of postmenopausal woman (17.4 ± 0.5 and 38.4 ± 1.7% conversion, respectively) and of men (27.1 ± 1.2 and 26.3 ± 26.3% conversion, respectively). In microsomes, the main metabolites were 17-hydroxylated 17α-OH-P and 17α-OH,20α-DH-P in renal cortex of both postmenopausal woman (32.1 ± 1.0 and 12.5 ± 1.2%, respectively) and men (31.5 ± 0.9 and 11.6 ± 1.4%, respectively). Ring-A reduction (5α-DH-P; 3β,5α-tetrahydro(TH)-P; 20α-DH,5α-DH-P) was detectable to a lesser extent in postmenopausal und male kidneys. The metabolism in mitochondrial and nuclear fractions of both sexes was similar in quality but not in quantity (10–18% conversion of P) to that in microsomes (up to 50% conversion of P).

On adding increasing concentrations of unlabelled P to human kidney cortex cytosol, the conversion of 4-^{14}C-P to 20α-DH-P decreased slightly but significantly. In cortex microsomes, the conversion to P metabolites was reduced to a greater extent at the highest concentration of added unlabelled P, but the conversion was still over 40% (Table 1).

Surprisingly, incubations with homogenates of premenopausal kidney cortex and medulla showed no 17-hydroxylated P metabolites. The conversion of P was higher in cortex than in medulla homogenates of pre-

Table 2
Percent conversion of ^{14}C-progesterone (200 000 cpm) in cortex and medulla homogenates of a premenopausal woman ($n = 5$)[a]

	20α-DH-P	5β-DH-P	5α-DH-P	3α,5β-TH-P	20α-DH,5α-DH-P	3β,5α-TH-P	3α,5α-TH-P 3β,5β-TH-P	20α-DH,3β,5α-TH-P	20α-DH,3α5α-TH-P	20α-DH,3β,5β-TH-P
Cortex	45.6 ± 2.6	0.5 ± 0.1	2.1 ± 0.4	1.6 ± 0.2	5.6 ± 0.6	1.7 ± 0.3	1.4 ± 0.3	2.6 ± 0.9	3.0 ± 0.8	
Medulla	9.3 ± 0.4	0.6 ± 0.1	3.4 ± 0.4	1.9 ± 0.3	1.1 ± 0.3	2.6 ± 0.3	2.1 ± 0.2	0.5 ± 0.2	0.6 ± 0.3	

[a] 300 mg homogenized tissue for each incubation was used, NADPH 10^{-3} mol/l as cosubstrate, 120 min incubation time. Means ± S.D. DH, dihydro, TH, tetrahydro, P, progesterone.

menopausal kidney (68.0 ± 3.1 and 22.6 ± 1.1%, respectively). The 20α-reduction of P was the major metabolic pathway, followed by 5α-reduction, and we could also detect small amounts of 5β- and 3α-reduced metabolites (Table 2).

4. Conclusions

In accordance with our hypothesis that progesterone (P) is metabolized in MC receptor target tissues, we proved that P is converted effectively to various metabolites in human renal tissue. Whereas the cytosol of postmenopausal and male kidneys showed only 20α-reduction activity, we found a metabolism to multiple metabolites in microsomes, where the conversion was very strong (up to 50% conversion). Surprisingly, we detected 17-hydroxylated P metabolites in postmenopausal and male kidneys, suggesting that 17α-hydroxylase is expressed in human postmenopausal and male kidney. In contrast, homogenates of the only premenopausal kidney that we were able to study formed no 17-hydroxylated P metabolites. We proved that incubations with increasing concentrations of P (Table 1) showed only a small decrease in percentage conversion due to saturation in metabolic activity, suggesting a very effective and potent enzyme system. We suggest that in postmenopausal and male kidneys, 17α-hydroxylase, 5α-reductase and 3β-reductase are involved in P metabolism. For 20α-reduction, there are several possible enzymes such as AKR1C1 (20α-HSD), AKR1C2, and AKR1C3 (17β-HSD type 5) (Lin et al., 1997; Dufort et al., 1999; Nishizawa et al., 2000). In homogenates of premenopausal female kidney the P metabolism was very high, and we also detected 5β-reductase and 3α-reductase activity.

Hence, this P metabolizing enzyme system could be an explanation for the weak MC-antagonist activity of P in vivo. Some other explanations have been put forward to explain this enigma (a) Only 3% of P is unbound to plasma proteins, whereas 37% of A is unbound and thereby available for intracellular action (Dunn et al., 1981); (b) the half-life of the aldosterone-MC receptor complex was estimated to be 600 min compared with 45 min of the progesterone-MC receptor complex (Souque et al., 1995); (c) P could be converted to the weak MC-agonist deoxycorticosterone (DOC) in MC target tissues, implying the idea of para- or autocrine formation of a MC receptor agonist at its site of action (Winkel et al., 1980; Casey et al., 1981). In our experiments, we found no formation of DOC from P in premenopausal, postmenopausal, or male human adult kidneys, even when we used high amounts of purified microsomal and cytosolic protein (12 and 100 mg, respectively; data not shown). Hence, the efficient renal enzyme activities described by these experiments are a possible mechanism of an enzyme-mediated MC receptor selectivity.

Acknowledgements

This work was supported by grant Dl 741/1-3 from the Deutsche Forschungsgemeinschaft (DFG).

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