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Effect of Angiotensin II on Rat Renal Cortical 11β-Hydroxysteroid Dehydrogenase

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Renal 11β-hydroxysteroid dehydrogenases (11β-HSDs) are subject to modulation by various endogenous factors. 11β-HSDs convert glucocorticoids into inactive 11-ketones and thereby determine tissue levels of active glucocorticoids and thus the extent of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) activation. As such, modulation of the activity of renal 11β-HSDs may contribute to the cascade of regulatory events involved in renal electrolyte water handling. We investigated whether renal 11β-HSDs are modulated by elevated circulating angiotensin II. In rats infused for 2 wk with angiotensin II (250 ng/[kg·min] subcutaneously), plasma angiotensin II, aldosterone, and corticosterone were raised 5.1-, 10.7-, and 2.3-fold, respectively, compared with control rats. Angiotensin II infusion raised corticosterone 11β-oxidation 1.46- and 1.35-fold in renal cortical proximal and distal tubules (enriched by Percoll centrifugation), respectively, but had no effect on 11β-HSD1 and 11β-HSD2 mRNA levels (semiquantitative reverse transcriptase polymerase chain reaction), except for distal tubular 11β-HSD1 mRNA, which was decreased to 50%. In vitro treatment of freshly isolated tubules with angiotensin II for 45 min prior to assessment of 11β-HSD activity showed no direct acute effects of angiotensin II on tubular corticosterone 11β-oxidation. The enhanced renal tubular corticosterone 11β-oxidation in vivo may partly protect renal GR and MR from elevated plasma corticosterone on angiotensin II infusion.

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Introduction

Mineralocorticoids and glucocorticoids play an important role in the regulation of the electrolyte- and water balance in the kidney. Apart from the fact that mineralocorticoids and glucocorticoids exert direct effects on renal transport proteins *(1–6)*, they modify the renal actions of other hormones involved in the regulation of renal transport processes. For instance, renal prostaglandin E_2 synthesis is decreased by both mineralocorticoids and glucocorticoids *(7)*, whereas mineralocorticoids synergistically increase rat renal collecting duct sodium reabsorption by vasopressin *(3)*, and glucocorticoids enhance the diuretic and natriuretic effects of atrial natriuretic peptide *(8)*.

The renal action of glucocorticoids is, to a great extent, determined by the presence of 11β-hydroxysteroid dehydrogenases (11β-HSDs). These enzymes catalyze the oxidation of the glucocorticoids cortisol (the major glucocorticoid in humans) and corticosterone (the major glucocorticoid in rats) into their inactive 11-ketone metabolites cortisone and 11-dehydrocorticosterone *(9–11)*. In contrast to the 11-ketones, cortisol and corticosterone bind with high affinities to glucocorticoid receptors (GRs) as well as mineralocorticoid receptors (MRs) *(9–12)*. Because 11β-HSDs inactivate glucocorticoids, intracellular levels of active glucocorticoids and thus the ability of glucocorticoids to interact with GRs and MRs depend on local 11β-HSD activity. By this mechanism, 11β-HSDs regulate the extent of GR activation *(11,13)*, protect the renal MR from activation by glucocorticoids, and maintain the MR specificity for aldosterone *(9–11)*. The importance of 11β-HSDs in controlling renal glucocorticoid action becomes evident in cases in which renal 11β-HSD is defective *(14–16)* or inhibited (*17,18*), resulting in sodium retention, hypokalemia, and hypertension owing to stimulation of (now freely acces-

sible) renal MRs and GRs. Furthermore salt-sensitive hypertension in humans *(19)* and in the Dahl rat strain *(20,21)* is associated with impaired renal glucocorticoid inactivation by 11β-HSD.

Two isozymes of 11β-HSD exist and both are found in rat kidney. 11β-HSD1 (the liver type 11β-HSD) is widely distributed throughout the body, has a relatively low affinity for natural glucocorticoids, and catalyzes glucocorticoid 11β-oxidation as well as 11-ketone reduction *(22,23)*. By contrast, 11β-HSD2 (the kidney type 11β-HSD) oxidizes natural glucocorticoids with high affinity (*24*) and is found in mineralocorticoid target organs *(24–26)*.

Because a change in the activity of 11β-HSDs in the kidney will indirectly modify renal electrolyte and water handling, knowledge of xenobiotic and endogenous factors that modulate renal 11β-HSDs is of great importance. Indeed, several studies have demonstrated regulation of renal 11β-HSDs in vivo, resulting in either increased *(27–33)* or decreased *(34–36)* 11β-HSD enzyme activity. Because modulation of renal 11β-HSDs may represent a possible mechanism, contributing to the cascade of regulatory events in renal physiology, we studied whether renal 11β-HSDs are modulated in response to angiotensin II. The idea behind this was that the actions of this important regulator of salt-water homeostasis and blood pressure *(37)* appear to be interrelated with glucocorticoids and mineralocorticoids. For instance, adrenal aldosterone and glucocorticoid syntheses are stimulated by angiotensin II *(38,39)*, whereas angiotensin II action in turn is enhanced by glucocorticoids *(40)*. The latter is also observed in the kidney, where angiotensin II–dependent ion transport is increased by glucocorticoids, an effect controlled by renal 11β-HSD *(41)*.

To study the possible effects of angiotensin II on renal 11β-HSDs, rats were infused for 2 wk with angiotensin II or vehicle (0.9% NaCl). Distal and proximal tubules were enriched by Percoll centrifugation to study corticosterone 11β-oxidation rates in freshly isolated intact tubular cells as well as 11β-HSD1 and 11β-HSD2 mRNA levels (by semiquantitative reverse transcriptase polymerase chain reaction [RT-PCR]). Acute effects of angiotensin II on renal tubular 11β-HSD activity were studied in freshly isolated tubular cells in vitro.

Results

Treatment of rats with angiotensin II for 14 d resulted in a 5.1-fold rise ($P < 0.05$) of this hormone in the circulation. Angiotensin II plasma levels were 42 ± 19 p*M* in control animals and 213 ± 82 p*M* in angiotensin II-treated animals (data as mean ± SEM), well within the range of previously published data *(42,43)*. Plasma aldosterone level were 1.5 ± 0.2 n*M* in control rats and 16 ± 3.0 n*M* in angiotensin II– treated rats, whereas corticosterone levels were 239 ± 57 n*M* in control rats and 558 ± 68 n*M* in angiotensin II-treated rats. Thus, the plasma levels of aldosterone and corticosterone were increased 10.7-fold ($P < 0.05$) and 2.3-fold ($P < 0.05$), respectively, by angiotensin II treatment.

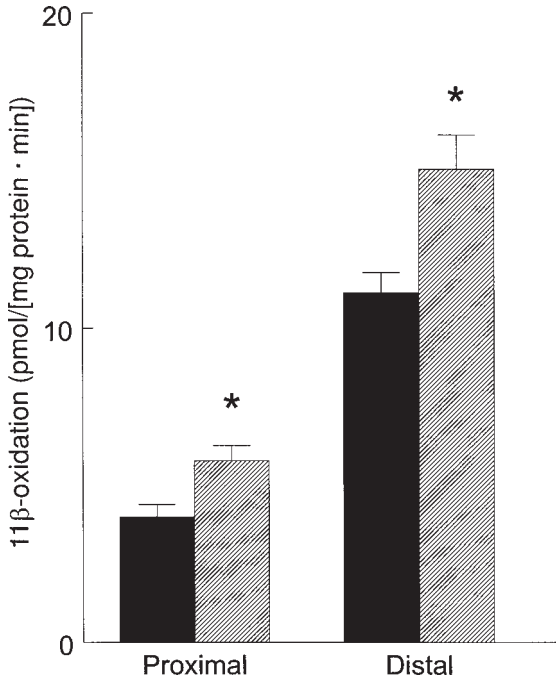

Fig. 1. Effect of angiotensin II infusion on rat renal proximal and distal tubular corticosterone 11β-oxidation.Freshly isolated proximal and distal tubular cells of rats infused with angiotensin II ($n = 10$) or 0.9% NaCl ($n = 13$) for 2 wk were incubated with corticosterone, following high-performance liquid chromatography (HPLC) analysis as described in Materials and Methods. Data are the mean corticosterone 11β-oxidation rates (pmol/ [mg protein·min]) ± SE. *Significantly different from control rats ($P < 0.01$). j , Control; W, angiotensin II.

Figure 1 shows that proximal and distal tubular inactivation of corticosterone by 11β-oxidation is significantly ($P < 0.01$) enhanced (1.46- and 1.35-fold, respectively) on angiotensin II treatment. We did not observe any reduction in 11-dehydrocorticosterone in intact proximal or distal tubular cells from untreated rats, consistent with other reports *(44,45)*. Also, in proximal and distal tubular cells from angiotensin II–treated rats, no 11-ketone reduction was detected.

Because proximal and distal tubular corticosterone 11β-oxidation were enhanced by angiotensin II, we now wished to test whether the expression of the 11β-HSD1 and 11β-HSD2 isozymes was changed by angiotensin II at the mRNA level.

Figure 2 shows the densitometric ratios of 11β-HSD1/RPS16 and 11β-HSD2/RPS16 PCR products. It is clear that angiotensin II had no significant effect on 11β-HSD1 mRNA levels in proximal tubular cells but reduced message levels in distal tubular cells to 50% of normal ($P < 0.05$). Regarding 11β-HSD2, angiotensin II appears to be without effect on mRNA levels in both cell types. We

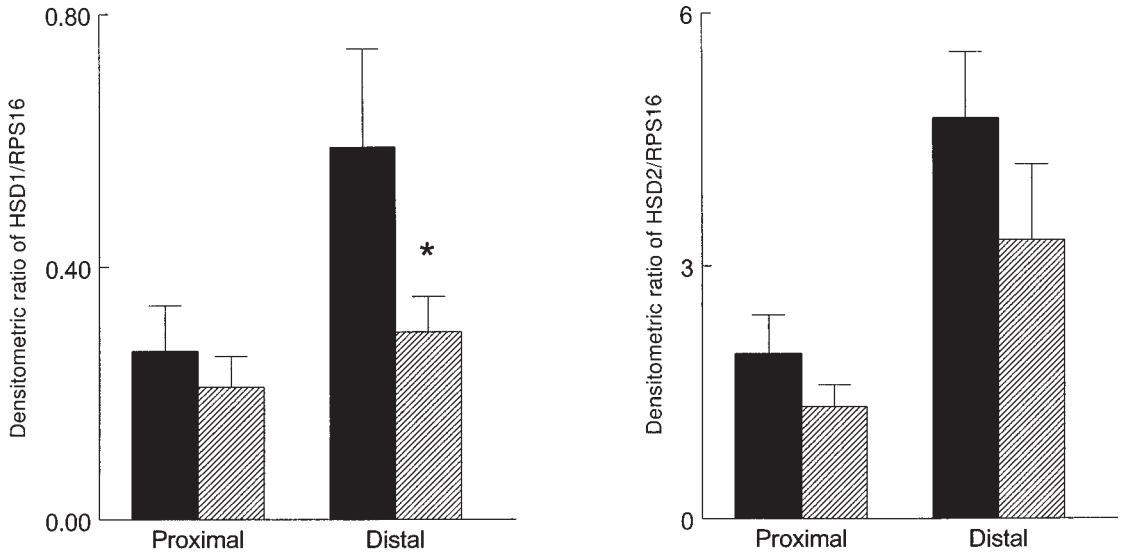

Fig. 2. Densitometric ratios of (**A**)11β-HSD1/RPS16 and (**B**)11β-HSD2/RPS16 for RNA obtained from proximal and distal tubular cells from control rats (j) and angiotensin II-treated (髟) rats. Data are the mean ± SE of nine rats (analyzed individually) in each group. *Significantly different from control rats ($P < 0.05$).

found 11β-HSD2 mRNA in proximal tubular cells, albeit at 2.4 levels times lower than in distal tubular cells. This finding is surprising because in *in situ* studies (*26,33*) 11β-HSD2 mRNA was found in rat distal but not proximal tubular cells, although distribution patterns appeared somewhat variable, showing high 11β-HSD2 mRNA levels in either rat renal outer cortex (*26*) or medulla (with low expression in the outer cortex) (*33*). Probably the 11β-HSD2 mRNA signals that we found in proximal tubules are attributable to contamination of proximal tubular mRNA with distal tubular or other renal cellular mRNA, owing to the cell separation method that we used.

As is clear from these findings, treatment of rats with angiotensin II enhances proximal and distal tubular corticosterone 11β-oxidation rates, but this is not reflected in increases in mRNA levels of the 11β-HSD isozymes. Therefore, we studied whether angiotensin II may exert direct acute effects on renal tubular 11β-HSD activity in vitro. Freshly isolated intact cells were incubated for 45 min with angiotensin II, before adding corticosterone and 15 min of additional incubation for the assessment of 11β-HSD activity. This approach was validated by testing the effects of the established 11β-HSD inhibitors glycyrrhetinic acid (*10,17*) and 11α-hydroxyprogesterone (*18*) as well as of vasopressin, which (at relatively high concentrations) raises cortical collecting duct 11β-HSD activity (*28*). As shown in Table 1, both glycyrrhetinic acid and 11α-hydroxyprogesterone decreased proximal and distal tubular corticosterone 11β-oxidation in a concentration-dependent manner ($P < 0.05$), whereas the highest concentration of vasopressin increased distal tubular corticosterone 11β-oxidation ($P < 0.05$). However, for angiotensin II, no effect was observed on either proximal or distal tubular corticosterone 11β-oxidation. Pilot experiments showed similar results at other angiotensin II concentrations.

Discussion

Local concentrations of 11β-hydroxyglucocorticoids are controlled by 11β-HSDs, which convert these compounds into inactive 11-ketones (*9–11*). In the kidney, this mechanism is responsible for the preservation of specificity of the MR for its actual ligand aldosterone as well as for the determination of the level of GR activation (*9–11,13*). Therefore, 11β-HSDs are an important factor in maintaining electrolyte and water homeostasis. Renal 11β-HSDs appear to be modulated by endogenous factors (*27–36*), and an activity shift in enzymes will lead to a change in renal GR and MR receptor activation. Because angiotensin II is an important regulator of volume homeostasis and renal sodium excretion (*37*), we wished to test whether elevated angiotensin II in the circulation has an effect on renal 11β-HSDs.

The findings in the present study show that on angiotensin II administration, both distal and proximal tubular corticosterone 11β-oxidation were enhanced (Fig. 1). This rise in 11β-HSD activity was not accompanied by a rise in 11β-HSD1 or 11β-HSD2 mRNA levels (Fig. 2). In fact, distal tubular 11β-HSD1 mRNA was decreased by 50%. Other studies in which the regulation of 11β-HSD was investigated (*27,33–36,46*) have shown that the relationship among 11β-HSD1 and 11β-HSD2 activities, protein levels, and mRNA levels is relatively complex and that changes at the mRNA, protein, and activity levels are not necessarily concomitant. Therefore, it is not surprising that we found angiotensin II to increase rat renal proximal and distal tubular 11β-HSD activity, without a rise in 11β-HSD1 or 11β-HSD2 mRNA.

Table 1
Effects of Angiotensin II, Vasopressin, and 11β-HSD Inhibitors Glycyrrhetinic Acid and 11α-Hydroxyprogesterone on Proximal and Distal Tubular Corticosterone 11β-Oxidation Rates In Vitro[a]

	Relative corticosterone 11β-oxidation rates (% of those of untreated cells)	
	Proximal tubules	Distal tubules
Glycyrrhetinic acid (10^{-7} *M*)	97 ± 5	99 ± 8
Glycyrrhetinic acid (10^{-5} *M*)	64 ± 4[b]	62 ± 12[b]
11α-Hydroxyprogesterone (10^{-7} *M*)	48 ± 2[b]	61 ± 8[b]
11α-Hydroxyprogesterone (10^{-5} *M*)	12 ± 5[b]	10 ± 10 [b]
Vasopressin (10^{-8} *M*)	103 ± 7	91 ± 7
Vasopressin (10^{-6} *M*)	110 ± 8	123 ± 7[b]
Angiotensin II (10^{-7} *M*)	96 ± 3	107 ± 9
Angiotensin II (10^{-5} *M*)	108 ± 10	104 ± 8

[a]Freshly isolated cells were incubated for 45 min at 37°C in the absence or presence of a variety of test substances. Subsequently corticosterone was added to the incubation mixtures, following another 15 min of incubation to assess corticosterone 11β-oxidation, as described. Data are means ± SEM.

[b]Significantly different from untreated cells ($P < 0.05$).

To investigate whether the 11β-HSD isozymes may have been changed at the protein level, we attempted to measure 11β-HSD1 and 11β-HSD2 protein levels by use of Western blotting. We found that distal tubular 11β-HSD2 protein levels tended to be decreased in angiotensin II-treated rats, but this difference was not significant, because of considerable variation within the angiotensin II group. For 11β-HSD1, detected signals were too low to make an accurate estimation of the 11β-HSD1 levels, and the amount of cellular material was too limited to achieve satisfactory signals by means of scaling up. Although for the aforementioned reasons it is difficult to attach a meaning to this, proximal tubular 11β-HSD1 protein levels tended to be higher in the angiotensin II–treated animals in these limited Western blotting experiments. Therefore, it is possible that 11β-HSD1 protein levels may have been elevated in the angiotensin II–treated rats. However, to determine whether this is indeed the case and to establish what causes the observed rise in proximal and distal tubular 11β-HSD activities, more detailed experiments are needed.

It is known that renal 11β-HSD activity can be enhanced by, e.g., vasopressin *(28)* by rapid mechanisms, other than an increase in total 11β-HSD protein, probably involving enzyme activation. To test whether a similar phenomenon might occur for angiotensin II, we studied the acute in vitro effect of angiotensin II on corticosterone 11β-oxidation rates of freshly isolated rat renal proximal and distal tubular cells. We confirmed that vasopressin rapidly enhances distal tubular 11β-HSD activity in vitro, but we found no effect of angiotensin II on either distal or proximal tubular corticosterone 11β-oxidation rates (Table 1). In line with these observations is the finding of a previous study, in which angiotensin II was shown to have no acute effect on 11β-HSD activity in renal medulla of normally fed rats in vitro (*32*). Exposure of the human colon cell line SW-620 to angiotensin II for 4 and 16 h generally also had no effect on 11β-HSD activity, although in two cases (4 h exposure to 2×10^{-6} *M* and 16 h exposure to 10^{-5} *M* angiotensin II), a decrease in 11β-HSD activity was observed, but there was no clear dose-response relationship *(47)*.

On the other hand, there is evidence that angiotensin II may decrease 11β-HSD activity in a rapid manner if intracellular angiotensin II levels are low. First, exogenous angiotensin II reduced renal medullary 11β-HSD activity in vitro when rats were fasted (which is expected to reduce intracellular angiotensin II levels) for 24 h prior to sacrifice but not when rats were fed *(32)*. Second, angiotensin-converting enzyme (ACE) inhibitors elevated 11β-HSD activity in vitro in renal medullas of fed rats only *(32)*. An enhancement of 11β-HSD activity by ACE inhibitors was also found in SW-640 cells *(47)*. The latter observations may indicate that a reduction in intracellular angiotensin II by ACE inhibitors leads to an elevation of 11β-HSD activity *(32)*. Taken together, these observations and the data in Table 1 suggest that it is quite unlikely that the elevated 11β-HSD activity seen on treating rats for 14 d with angiotensin II in vivo can be explained by direct acute (but long-lasting) effects of circulating angiotensin II. The rise in 11β-HSD activity following 14 d of angiotensin II infusion in vivo, rather appears to be attributed to relatively slow and possibly indirect (e.g., via glucocorticoids, mineralocorticoids, or other hormones or factors that are changed by angiotensin II) effects of circulating angiotensin II. Clearly, to determine which mechanism is responsible for the

observed rise in corticosterone 11β-oxidation (i.e., angiotensin II itself or factors modified by angiotensin II) and to discern between acute and long-term effects of angiotensin II, further study is necessary.

Administration of angiotensin II elevated plasma corticosterone levels about 2.3-fold (Table 1). In parallel, the capacity of 11β-HSDs to inactivate corticosterone was raised 1.46- and 1.35-fold in proximal and distal tubular cells, respectively. Therefore, rat renal cortical proximal and distal tubular cells will at least be partly protected for the elevated corticosterone plasma levels because of the increased inactivation of corticosterone by 11β-HSD. This may blunt a possible increase in proximal and distal tubular GR activation, owing to the elevation in corticosterone levels by angiotensin II.

Similarly, the rise in distal tubular corticosterone 11β-oxidation may, to some extent, contribute to the protection of distal tubular MRs for the elevated corticosterone levels and thereby maintain the specificity of these receptors for aldosterone, the level of which is raised by angiotensin II. We recently found that on feeding rats a diet with high potassium chloride content, a condition in which aldosterone is raised, distal tubular 11β-HSD activity and 11β-HSD2 protein were elevated *(31)*. Furthermore, in rats on a low-sodium diet, in which aldosterone levels were elevated, kidney microsomal NAD-dependent 11β-HSD activity was increased by a nongenomic mechanism (*33*). Finally, aldosterone itself has been reported to increase rat cortical collecting duct 11β-HSD activity *(28)*. Because glucocorticoids exert potent mineralocorticoid effects if the MR is not protected by 11β-HSD *(14–18)*, it seems contradictory that glucocorticoids are excluded more extensively from binding to the MR by 11β-HSD, under conditions in which aldosterone is raised. However, transfected MRs regulate gene transcription more efficiently in the presence of aldosterone than in the presence of active glucocorticoids, in spite of similar ligand affinities *(48,49)*, whereas the ligand-receptor complex is more stable for aldosterone than for glucocorticoids *(48)*. This may indicate that aldosterone is a more efficient agonist for the MR than glucocorticoids. Thus, it may well be that elevated distal tubular 11β-HSD activity is important for optimal activation of the MR under physiologic conditions in which aldosterone is to be raised.

Materials and Methods

Treatment of Animals

Experiments were conducted according to institutional guidelines and were approved by the local ethical committee for the use of experimental animals. Male Wistar rats (280–350 g) (Iffa Credo, Someren, Netherlands) were treated for 2 wk with angiotensin II (Sigma, St. Louis, MO) (used at a dose of 250 ng [kg · min] $n = 10$ rats) or vehicle (0.9% [w/v] NaCl; $n = 13$ rats), using osmotic minipumps (Alzet 2002; Alza Co, Palo Alto, CA) that were placed subcutaneously in the neck under ether anesthesia *(42,43)*. This angiotensin II dose was chosen, because it is known from other studies at our department *(42)* to produce a clear rise in angiotensin II plasma levels. Angiotensin II is known to be stable for at least 2 wk in implanted osmotic minipumps *(43)*. Rats were randomly assigned to the experimental groups. There were no significant differences in body weights between the groups before and after the treatments.

Determination of Corticosterone, Aldosterone, and Angiotensin II in Plasma

Using EDTA plasma, aldosterone and angiotensin II were determined by radioimmunoassays *(50,51)*, and corticosterone was determined by HPLC after conversion of corticosterone and cortisol (added as an internal standard) to fluorescent products with sulfuric acid, as described by Mason et al. *(52)*.

Isolation of Cells and Determination of Corticosterone 11β-Oxidation

Rats were anesthetized with sodium-pentobarbitone (60 mg/kg, intraperipentoncally) and the kidneys were removed. Isolation of cortical tubular cells by collagenase/hyaluronidase digestion of cortex pieces and separation of proximal and distal tubular cells by Percoll density gradient centrifugation were conducted as previously described *(31)*. After prewarming solutions, freshly isolated cells, corresponding to proximal and distal tubular protein amounts of 0.2 and 0.1 mg, as determined by the method of Smith et al. (*53*), were incubated for 15 min at 37°C with 100 n*M* corticosterone (Sigma) in 1 mL of modified Krebs-Henseleit buffer (118 m*M* NaCl, 4 m*M* KCl, 1.0 m*M* KH_2PO_4, 27 m*M* $NaHCO_3$, 0.12 m*M* $MgCl_2$, 5 m*M* glucose, 1.2 m*M* $MgCl_2$, 1.25 m*M* $CaCl_2$, and 11.5 m*M* HEPES brought to pH 7.4 with NaOH. Pilot experiments showed that at this incubation time and proximal and distal tubular protein amount, 11-dehydrocorticosterone formation was within the linear range. A substrate concentration of 100 n*M* was chosen, because it yields readily measurable 11-dehydrocorticosterone levels and is not too far above the free corticosterone concentrations observed in normal rats *(54)*. After stopping the reactions with 100 μL of 1 *M* o-phosphoric acid and adding 100 pmol of cortisone as internal standard, steroids were extracted with 6 mL of methylene chloride and analyzed on HPLC. The HPLC system consisted of a 125 × 4 mm Nucleosil C18 column (Macherey-Nagel, Düren, Germany) as the stationary phase and a (helium degassed) 1/320/680 (v/v/v) mixture of trifluoroacetic acid/acetonitrile/milli-Q water as the mobile phase. The flow rate was 1.2 mL/min (SP8810 pump; Spectra Physics, San Jose, CA), and steroids were detected at 243 nm (SP8490 detector; Spectra Physics). Peak area ratios (vs internal standard) were calculated using a Hitachi D2000 (Tokyo, Japan) integrator and compared with calibration curves.

Back-conversion of 11-dehydrocorticosterone to corticosterone by 11-ketone reduction was assessed in a similar manner.

We used intact tubules for assessing 11β-HSD activities, in order to avoid changes in enzyme activity owing to homogenization processes and to avoid the need for adding external cofactors.

Effects of Angiotensin II and Some Known Modulators of Renal 11β-HSD in Vitro

Immediately after isolation, proximal and distal tubular cells were incubated for 45 min at 37°C in the absence (controls) or presence of glycyrrhetinic acid, 11α-hydroxyprogesterone (Sigma), angiotensin II (10^{-7} and 10^{-5} *M*), or 10^{-8} and 10^{-6} *M* arginine vasopressin (Neosystem, Strassbourg, France) in 1 mL of modified Krebs-Henseleit buffer (*see* previous section), supplemented with 2% (w/v) bovine serum albumin, 1 vol% (100X) minimum essential medium (MEM)-vitamins (Gibco-BRL), 2 vol% (50X) MEM-amino acids (Gibco-BRL), 5 m*M* sodium pyruvate, 1 m*M* glycin, and 2 m*M* lactic acid. Next, 25 μL of a prewarmed corticosterone solution was added to obtain a substrate concentration of 100 n*M,* and the cells were incubated for another 15 min to determine corticosterone 11β-oxidation as described above. For the substances to be tested, four to six cell preparations were used (in duplicate or triplicate). Corresponding controls (without test substance) were incubated simultaneously.

RT-PCR Analysis of 11β-HSD1 and 11β-HSD2 mRNA

Semiquantitative RT-PCR was conducted as previously desribed in greater detail *(31)*. Briefly, total RNA of the renal cells was isolated using the Quick-Prep procedure (Pharmacia, Uppsala, Sweden) and treated with DNase I. First-strand cDNA synthesis was performed by transferring 25 ng of heat-denatured RNA and 200 pmol of random hexamer primer to a Ready-To-Go first-strand bead (Pharmacia) tube, adjusting the end volume to 33 μL with milli-Q water and 1 h of incubation at 37°C. The RT reaction was stopped by heat inactivation of the transcriptase, and samples were cooled to 4°C. Subsequent PCR of freshly synthesized cDNA (corresponding to 188 pg of RNA) was conducted using Ready-To-Go PCR beads (Pharmacia) and 75 pmol of the 5'- and 3'-primers in a reaction volume of 25 μL. After adding a droplet of mineral oil and denaturation, samples were incubated for 35 cycles at 64°C (45 s), 72°C (90 s), and 95°C (30 s), followed by a 15-min extension. PCR primers (Pharmacia) were selected on the basis of published cDNA sequences (using the Internet databases and search tools accessible at and via the site of the National Center for Biotechnology Information of the National Institutes of Health) and had the following sequences (5'→3'):

1. (5'-primer 11β-HSD1) GAGTTCAGACCAGAAATGCTCC ,
2. (3'-primer 11β-HSD1),TGTGTGATGGATTGAGAATGAGC.
3. (5'-primer 11β-HSD2),GATGTTCCCCTCGCCTGAA.
4. (3'-primer 11β-HSD2) ATGAGCAGTGCAATAGCTGCCTTG
5. (5'-primer RPS16) AAGTCTTCGGACGCAAGAAAA
6. (3'-primer RPS16) CAAAGGTAAACCCTGATCCTTGAG.

The resulting PCR products have fragment lengths of 290, 348, and 439 bp for 11β-HSD1, 11β-HSD2, and RPS16 respectively. For every sample, PCRs of all primer sets (in different test tubes) were carried out in parallel, and PCR products were analyzed by nondenaturating polyacrylamide gel electrophoresis (4.5% T), following silver staining and densitometric determination of the ratios of signals of 11β-HSD1 and 11β-HSD2 vs signals of RPS16, used as the internal control for RNA integrity and reverse transcription efficiency *(55)*. Densitometric signals of the PCR products increased linearly with the amount of RNA (tested in the range of 0–200 ng) initially used in first-strand synthesis.

Statistical Analyses

Statistical analyses were performed using the statistical software program INSTAT (GraphPAD Software, San Diego, CA). Differences between experimental and control animals were tested for significance by unpaired Student's *t*-tests. Regarding plasma hormone levels, differences between the animals were tested using the Mann-Whitney *U*-test. Effects of the compounds on cellular 11β-HSD activity in vitro were tested for significance by analysis of variance and subsequent calculation of the least significant differences test *(56)*.

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