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Characterization of 11β-Hydroxysteroid Dehydrogenase Activities in the Renal Cell Line LLC-PK$_1$

Evidence for a Third Isoform?

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We studied 11β-hydroxysteroid dehydrogenase activities in the renal cell line LLC-PK$_1$ and the effects of different steroids on them. Cortisol was oxidized in the presence of NAD as well as NADP, reflecting the presence of two different 11β-HSD forms. Enzyme kinetics for cortisol 11β-oxidation were: V_{max} = 5.9 pmol/(min × mg), K_m = 0.2 μ*M* with NAD, and V_{max} = 4.5 pmol/(min × mg), K_m = 1.0 μ*M* with NADP. Interestingly, no reverse reaction was observed when using cortisone and NADPH as substrate and cosubstrate, respectively. Exposure of cells to a variety of steroids had different effects on cortisol 11β-oxidation rates with NADP compared to those with NAD. Dexamethasone initially (3–60 min of exposure) decreased the NAD-dependent 11β-HSD activity to about 60%, which was no longer evident after 2 h or longer. By contrast, the 11β-oxidation of cortisol with NADP increased by dexamethasone treatment of the cells, after a lagtime of about 2 h, and this effect was still evident after 32 h. The increase of 11β-HSD activity with NADP by dexamethasone was concentration dependent (estimated EC$_{50}$: 125 n*M*). The antiglucocorticoid RU486 did not antagonize dexamethasone induction. Exposure of cells for 19 h to 1 μ*M* cortisol, cortisone, progesterone, and estradiol also increased NADP-dependent cortisol 11β-oxidation, but had no effect on the NAD-dependent 11β-HSD activity. Immunoblot and reverse transcriptase-polymerase chain reaction experiments failed to detect any 11β-HSD 1 protein or mRNA in these cells. Our observations suggest that in LLC-PK$_1$ cells, two forms of 11β-HSD exist, which differ in cosubstrate dependency, kinetics for cortisol, and modulation by steroids. Whereas the NAD-dependent form seems identical to renal 11β-HSD 2, the NADP-dependent 11β-HSD possibly resembles an as yet unknown third isoform.

Key Words: 11β-Hydroxysteroid dehydrogenase; LLC-PK$_1$ cells; cortisol metabolism; dexamethasone induction.

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Introduction

11β-Hydroxysteroid dehydrogenase (11β-HSD) isozymes catalyze the dehydrogenation of the naturally occurring glucocorticoids cortisol and corticosterone to their inactive 11-keto products cortisone and 11-dehydrocorticosterone, which, unlike the 11β-alcohols, are not able to bind to corticosteroid receptors *(1–4)*. At present, it is well established that 11β-HSDs act as pre-receptor control devices, by determining levels of active/inactive glucocorticoid concentrations, thereby maintaining the aldosterone specificity of mineralcorticoid receptors (MRs) as well as regulating the extent of glucocorticoid receptor (GR) stimulation *(2,4–6)*. Two types of 11β-HSD have been identified *(7–12)*. The type 1 isozyme of 11β-HSD (11β-HSD 1) is a low-affinity NADP/NADPH-dependent dehydrogenase/oxoreductase, with an apparent K_m in the micromolar range. The predominant role of this isozyme in vivo has been suggested to be 11-oxoreduction, i.e., the generation of active glucocorticoid *(13,14)*.

By contrast, type 2 11β-HSD (11β-HSD 2) is a high-affinity, unidirectional, NAD-dependent dehydrogenase (apparent K_m in the nanomolar range) that is supposed to protect the MR from cortisol excess. A defect or inhibition of this enzyme system has great (pathological) implications, leading, among other things, to hypertension *(5,6,15)*. Mutations in the gene encoding 11β-HSD 2 are responsible for a heritable form of hypertension, apparent mineralocorticoid excess, in which cortisol acts as a potent mineralocorticoid *(16–18)*.

Both enzymes are located in the endoplasmic reticulum, but their distribution throughout the body is different *(7,10–*

12,19). 11β-HSD 1 is found ubiquitously and is often colocalized with GRs, whereas 11β-HSD 2 is expressed predominantly in mineralcocorticoid target tissues such as the kidney and colon, and is often colocalized with MRs. Therefore, 11β-HSD 1 has been stated to regulate GR-ligand interactions, whereas 11β-HSD 2 is believed to maintain primarily the aldosterone specificity of MRs *(12,15)*.

In a recent study, Leckie et al. *(20)* demonstrated the presence of MRs and GRs in the renal cell line LLC-PK$_1$ and confirmed previous findings *(21)* that these cells possess 11β-HSD activity. They showed that this activity is owing to an NAD-dependent 11β-HSD 2 type of enzyme that is able to protect (plasmid expressed) MRs from interaction with corticosterone.

In the present study, we used LLC-PK$_1$ cells as a model to investigate the effects of different steroids (with special focus on corticosteroids and female sex hormones) on 11β-HSD activity, to provide insight into the modulation of 11β-HSD in response to internal or external factors (e.g., elevated steroid hormone levels). Interestingly, we found two different 11β-HSD activities in LLC-PK$_1$ homogenates, one with NAD and one with NADP as cosubstrate. For both activities, only 11β-dehydrogenation was observed, which is remarkable since usually NADP/NADPH-dependent 11β-HSD 1 activity is reversible. Moreover, in addition to exhibiting different K_m values for corticol 11β-oxidation, the modulation by steroids was operative exclusively with the NADP- dependent but not with the NAD-dependent 11β-HSD isoform. Our results are indicative of the existence of two 11β-HSD isoforms in the renal cell line LLC-PK$_1$, of which the NADP-dependent form apparently is different from the well-characterized 11β-HSD 1, and possibly resembles an as-yet unknown third isoform.

Results

At cosubstrate and cortisol concentrations of 1 m*M* and 1 μ*M*, respectively, cortisol was oxidized at its 11β-hydroxy group at approximately three to four times higher rates with NAD than with NADP in cell homogenates. No cortisol 11β-oxidation was detected in cell homogenates when no cosubstrate was added. Nonlinear regression analysis of substrate-velocity plots for 11β-oxidation of cortisol revealed V_{max} values of 5.9 and 4.5 pmol/(mg × min), and K_m values for cortisol of 0.2 and 1.0 μ*M* with NAD and NADP as cosubstrates, respectively (Fig. 1).

Extensive oxidation of cortisol and dexamethasone to their 11-ketone metabolites was also observed in whole cells. High-performance liquid chromatography analysis of medium extracts showed that if cells were exposed to cortisol for 19 h, all cortisol appeared to be converted to cortisone. Similarly, whereas the amount of dexamethasone in culture medium declined with exposure time (after 19 h only 60% of the originally added dexamethasone was left), the amount of

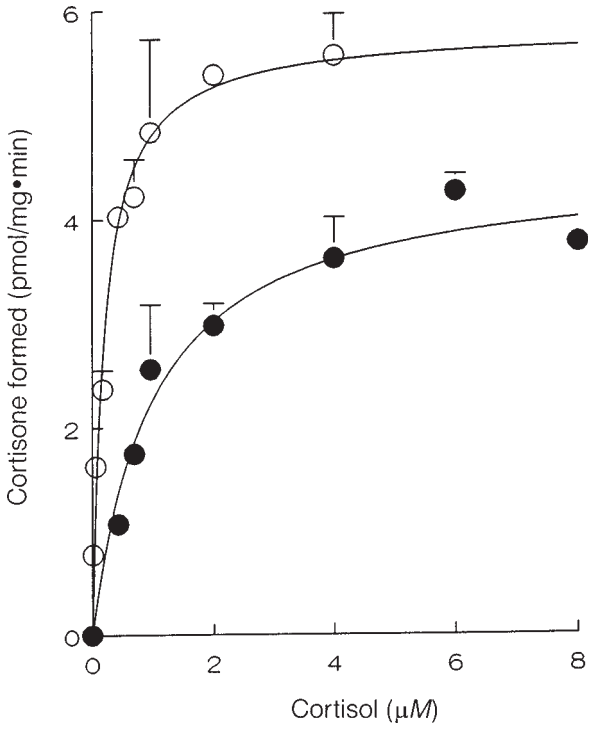

Fig. 1. Substrate-velocity plots of the (○) NAD- and (●) NADP-dependent 11β-oxidation of cortisol in homogenates of LLC-PK$_1$ cells. Data are expressed as means and standard errors of two independent experiments. Assays were performed in duplicate. Curve fitting was performed using the GPAD program (GraphPAD Software, San Diego, CA).

a substance, that coeluted with a 11-keto-dexamethasone standard (obtained by oxidation of dexamethasone-21-acetate and subsequent hydrolysis of the 11-dehydroproduct) inclined in parallel (data not shown). Nevertheless, in whole cells as well as in homogenates (after adding NADPH or NADH as cosubstrates), we did not detect any 11-ketone reduction of cortisone.

In studying the effect of steroids on 11β-HSD, a first set of experiments was directed to determine the time dependency of the effect of dexamethasone on the 11β-oxidation of cortisol, as summarized in Fig. 2. Figure 2 shows that the NAD-dependent 11β-HSD activity is affected differently by treatment of the cells with dexamethasone than the 11β-HSD activity found when NADP is used as a cosubstrate. Although showing an initial decrease of cortisol 11β-oxidation with NAD at short-term (< 2 h) dexamethasone exposure ($p < 0.05$), on longer exposure to dexamethasone (2–32 h), this activity was not different from that in untreated cells. By contrast, 11β-oxidation rates of cortisol with NADP were on average 1.8-fold higher ($p < 0.05$) after 2–32 h of dexamethasone exposure.

To study the observed incline of NADP-dependent 11β-HSD activity in greater detail, further experiments were performed in which LLC-PK$_1$ cells were exposed to different concentrations of dexamethasone for 19 h, an exposure time at which dexamethasone enhanced cortisol 11β-oxi-

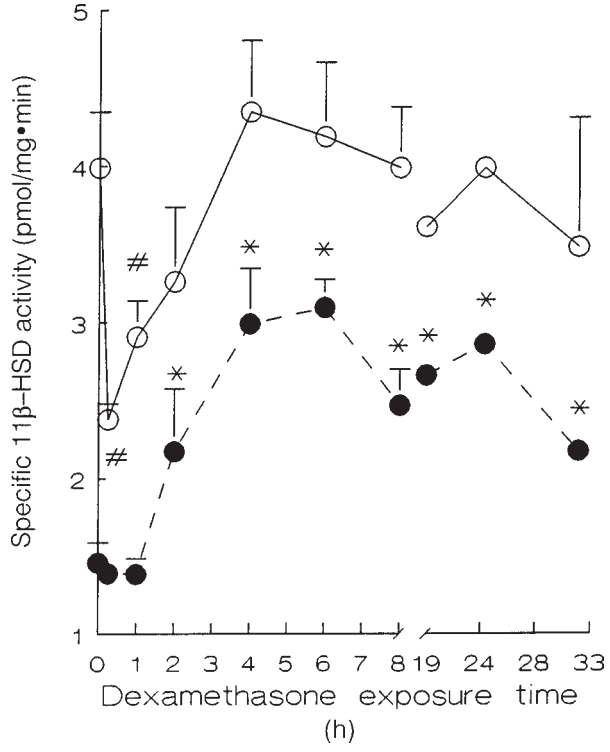

Fig. 2. Time-dependent effects of dexamethasone treatment on the (—○—) NAD- and (—●—) NADP-dependent 11β-oxidation of cortisol in LLC-PK$_1$ cells. LLC-PK$_1$ cells were treated for different periods of time with 1 μ*M* dexamethasone before cells were harvested and homogenized and their cortisol 11β-oxidation activity determined as described in Materials and Methods. Data are expressed as means and standard errors of three to four independent experiments. Assays were performed in duplicate. *and #, Significantly different ($p < 0.05$; analysis of variance [ANOVA], LSD) from controls for NADP- and NAD-dependent 11β-HSD, respectively.

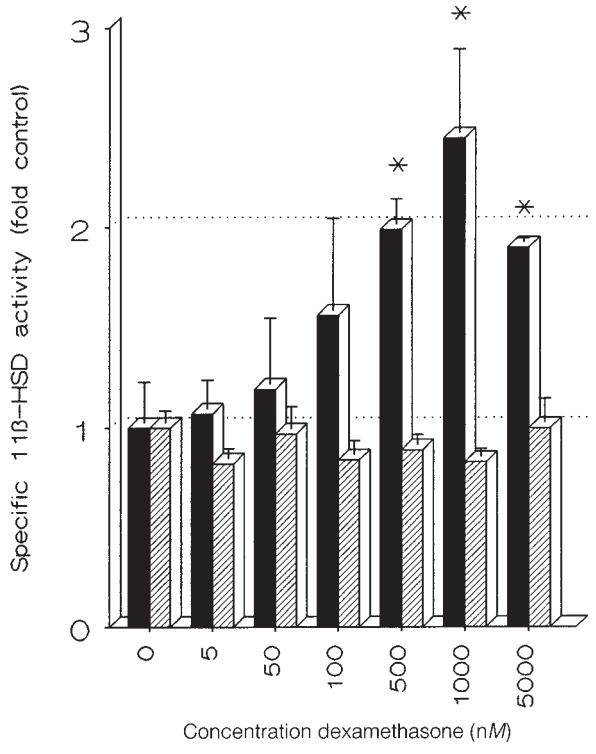

Fig. 3. Concentration dependency of the effect of treating LLC-PK$_1$ cells for 19 h with dexamethasone on 11β-HSD activities. Data are expressed as means and standard errors of two independent experiments and represent specific cortisol 11β-oxidation rates (relative to control values) in cellular homogenates. Assays were performed in duplicate. *,Significantly different ($p < 0.05$; ANOVA, LSD) as compared to controls for the NADP-dependent 11β-HSD activity. ■, NADP; ▨ NAD.

dation rates with NADP approx 1.8 fold (Fig. 2). As depicted in Fig. 3, the increase in 11β-HSD activity with NADP was dependent on the concentration of dexamethasone, showing a maximum effect of induction at 1 μ*M*, and an estimated EC_{50} for dexamethasone of 125 n*M*. In contrast to what is observed for NADP, no effects were observed on NAD-dependent 11β-HSD activity in the range of dexamethasone concentrations tested.

Fig. 4 shows the effect on 11β-HSD activity of treating LLC-PK$_1$ cells for 19 h with a variety of steroids. Next to dexamethasone, cortisol, cortisone, progesterone, and β-estradiol enhanced the 11β-HSD activity with NADP 1.5 to 1.8-fold ($p < 0.05$). Aldosterone also increased NADP-dependent 11β-HSD activity, but this change did not reach statistical significance. The enhancement of cortisol 11β-oxidation by dexamethasone was not influenced by the glucocorticoid/progesterone antagonist RU486 (mifepristone), which itself did not have an effect on 11β-HSD activity. Similar observations were made with 10 times higher RU486 concentrations (data not shown). However, none of the steroids exerted any significant changes in the 11β-HSD oxidation rates if NAD was used as cosubstrate. This disparity in the modulation of 11β-HSD activities with NADP compared to those with NAD in LLC-PK$_1$ cells is underlined by the fact that omission of fetal calf serum (FCS) in the culture medium of the cells for 48 h decreased cortisol 11β-oxidation activities in cellular homogenates with NADP to 50%, but was without effect on cortisol conversion rates with NAD (Fig. 4).

In immunoblot experiments, we failed to detect any immunoreactive protein with an antibody directed toward 11β-HSD 1 from mouse liver (Fig. 5). This polyclonal antibody has previously been shown to crossreact with 11β-HSD 1 from a variety of species, including chicken, guinea pig, rat, sheep, rabbit, pig, and human *(22)*. Compared to mouse liver microsomal 11β-HSD 1, which served as positive control (lane 2 in Fig. 5), no band was observed in LLC-PK$_1$ homogenates, even when the cells were taken from induction experiments with dexamethasone and when a sensitive chemiluminescence detection method was applied. These results are indicative of the absence of 11β-HSD 1 protein in LLC-PK$_1$ cells.

Moreover, no signal of 11β-HSD 1 mRNA could be obtained in reverse transcriptase- polymerase chain reac-

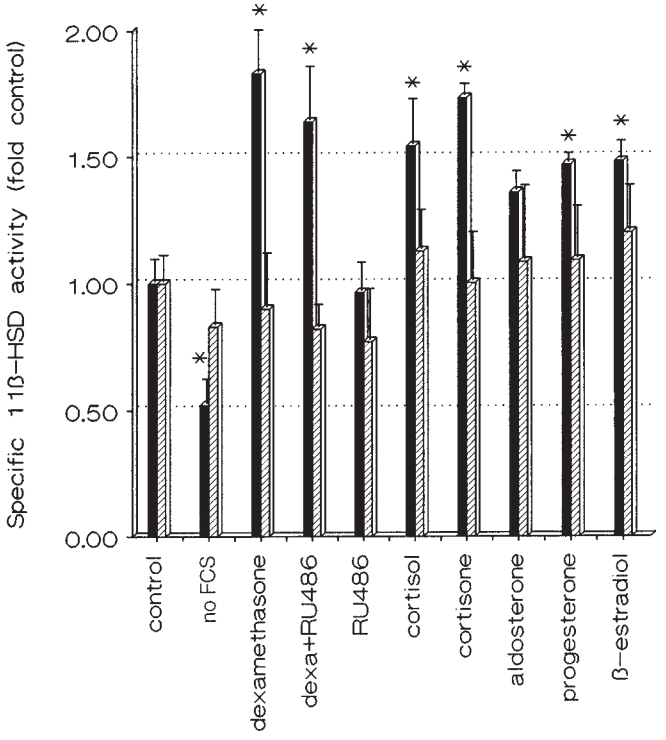

Fig. 4. The effect of treating LLC-PK$_1$ cells for 19 h with various steroids or of omission of FCS for 48 h in culture medium on cortisol 11β-oxidation rates. Data are expressed as means and standard errors of three to nine independent experiments and represent specific cortisol 11β-oxidation rates (relative to control values) in cellular homogenates. Assays were performed in duplicate. *,Significantly different ($p < 0.05$; ANOVA, LSD) as compared to controls for the NADP-dependent 11β-HSD activity. ■, NADP; ▨ NAD.

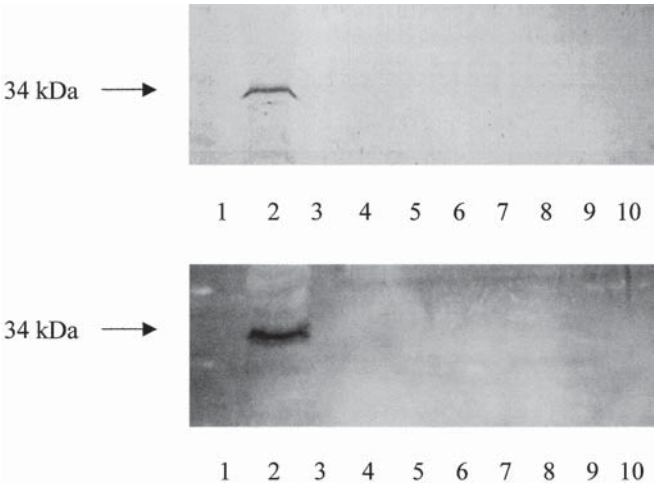

Fig. 5. Immunoblot of LLC-PK$_1$ cell homogenates. Polyclonal antibodies raised in rabbits against mouse 11β-HSD 1 were incubated as primary antibody. LLC-PK$_1$ cell homogenates after dexamethasone induction (19 h) were used as source of antigen. Antigen-antibody complexes were identified by the peroxidase reaction of the secondary conjugated antibody specific for rabbit IgG. Signals were developed by the chloronaphthol method **(top)** or by chemiluminescence detection **(bottom)**. Lane 1, blank (no protein applied); lane 2, mouse liver microsomes (positive control); lane 3, LLC-PK$_1$ cell homogenate (uninduced); lanes 4–10, LLC-PK$_1$ cell homogenate after induction with 0.005, 0.05, 0.1, 0.5, 1, 5, and 50 μ*M* dexamethasone, respectively. The arrow indicates 11β-HSD 1 from mouse liver microsomes at 34 kDa.

tion (RT-PCR) experiments with LLC-PK$_1$ cells (Fig. 6). For these studies, we used primers homologous to conserved regions of mouse and human 11β-HSD 1 cDNA. Notably, with primers homologous to human 11β-HSD 1 cDNA, we yielded a PCR amplicon of correct size (395 bp) with mouse RNA as template, in addition to respective controls (Fig. 6). Moreover, by applying primers homologous to 11β-HSD 2 *(20)*, a PCR amplification product of correct size (613 bp) was obtained. Taken together, these results confirm both the absence of 11β-HSD 1 and the presence of 11β-HSD 2 in LLC-PK$_1$ cells. Consequently, the NADP-dependent 11β-oxidation activity of cortisol to cortisone present in LLC-PK$_1$ cells seems to be affiliated with a currently unknown third isoform of 11β-HSD.

Discussion

The ability of glucocorticoids to exert effects via cytosolic MRs and GRs is, among other things, dependent on the presence of 11β-HSDs, since these enzymes determine local levels of active 11β-hydroxyglucocorticoids *(2,5)*. In this respect, renal LLC-PK$_1$ cells have recently been suggested to represent a valuable tool in studying the role of 11β-HSD 2 in preventing glucocorticoids from binding to mineralocorticoid receptors *(20)*. In agreement with an earlier study *(21)*, a relatively high 11β-HSD activity in these cells was found that was attributed to the action of 11β-HSD 2 because of the observed cosubstrate preference for NAD, the low K_m values for glucocorticoids, and an extracted mRNA with high sequence identities compared to that of 11β-HSD 2 *(20)*. In accordance with these studies *(20,21)*, we found 11β-HSD activity in LLC-PK$_1$ cell homogenates with NAD. In addition, we detected considerable 11β-dehydrogenase activity with NADP as cosubstrate. Detailed kinetic analyses revealed that the K_m for cortisol 11β-oxidation was higher when NADP, rather than NAD, was used.

Surprisingly, no back-conversion of cortisone to cortisol by 11-ketone reduction was observed when LLC-PK$_1$ cell homogenates were incubated with cortisone and NADPH as substrates. Similarly, in media of LLC-PK$_1$ cells, incubated for 19 h with cortisone, we could not detect any cortisol, indicating that the 11-ketone reductase activity is absent in LLC-PK$_1$ cells. This is remarkable, because NADP/NADPH-dependent 11β-oxidoreduction of glucocorticoids is known to be reversible and has been attributed to the action of 11β-HSD 1, the isoform with highest abundancies in liver. In fact, the main function of 11β-HSD 1 has been postulated to be the activation of 11-ketoglucocorticoids by acting as an NADPH-dependent cortisone reductase *(23)*. Furthermore, reductase activity has been shown to predominate in all cell lines transfected with 11β-HSD 1 cDNA *(24–26)*.

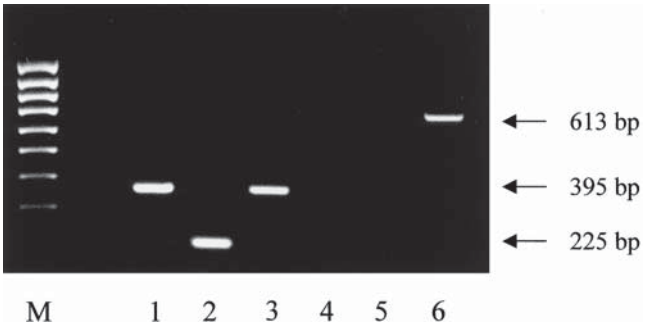

Fig. 6. RT-PCR studies on the mRNA expression of 11β-HSDs in LLC-PK$_1$ cells. The RT-PCR amplification products were resolved by agarose gel electrophoresis. Lane 1, human liver total RNA as template and primers homologous to human 11β-HSD 1 yielded the expected 395-bp fragment. Lane 2, mouse liver total RNA as template and primers homologous to mouse 11β-HSD 1 yielded the expected 225 bp fragment. Lane 3, the primers homologous to human 11β-HSD 1 recognized mouse liver 11β-HSD 1 mRNA, thereby resulting in the corresponding 395-bp amplicon. Lanes 4 and 5, neither the primers homologous to mouse 11β-HSD 1 nor those homologous to human 11β-HSD 1 recognized any 11β-HSD 1 mRNA in LLC-PK$_1$ cells. Lane 6, 11β-HSD 2 mRNA in LLC-PK$_1$ cells is recognized by primers homologous to 11β-HSD 2, thereby resulting in the expected 613-bp fragment. M, DNA ladder.

Treatment of LLC-PK$_1$ cells with a variety of steroids differentially influenced cortisol 11β-oxidation rates in the presence of NAD or NADP. Although short-term (<2 h) dexamethasone treament of the cells resulted in a decrease in cortisol 11β-oxidation with NAD, on longer exposure to dexamethasone (2–32 h), this activity was not different from that in untreated cells. This initial decrease in NAD-dependent cortisol oxidation can possibly be explained by a direct inhibitory action of the high initial (but rapidly declining) concentrations of dexamethasone, which is known to serve as a substrate and competitive inhibitor of 11β-HSD 2 *(10,27)*, in the medium. By contrast, NADP-dependent 11β-HSD activity was not altered by dexamethasone within the first 2 h, and was even significantly enhanced on longer exposure to dexamethasone.

Glucocorticoids have been shown to increase 11β-HSD activity both in vitro *(28,29)* and in vivo *(30,31)*. In human fibroblasts, this effect has been shown to be mediated via the GR *(28)*. However, the enhanced NADP-dependent 11β-HSD activity we observed upon exposure of LLC-PK$_1$ cells to dexamethasone is probably not a GR-specific effect, since the glucocorticoid antagonist RU486 did not inhibit rise in the activity by dexamethasone in agonist/antagonist ratios that would be expected to show complete inhibition of GR-mediated effects *(28,32)*. In addition, the EC_{50} of dexamethasone (about 125 n*M*) we found for the effect on NADP-dependent 11β-HSD activity by far exceeded its reported *Kd* of 0.3 n*M* for GRs in LLC-PK$_1$ cells *(20)*.

Furthermore, in addition to dexamethasone, treatment of LLC-PK$_1$ cells with cortisol, cortisone, progesterone, and estradiol led to an increase in the NADP-dependent 11β-HSD activity, but did not significantly influence cortisol 11β-oxidation with NAD. Remember here that cortisone is a proglucocorticoid that is not converted to active cortisol in LLC-PK$_1$ cells, as already discussed. Taken together, these observations suggest the rise in NADP-dependent 11β-HSD activity in LLC-PK$_1$ cells by steroids to be rather aspecific. Whether this effect is obtained by enhanced protein or mRNA synthesis (as has been described for the increase of 11β-HSD activity by glucocorticoids in e.g. refs. *28* and *29*) or by other mechanisms, such as activation of latent enzyme, remains to be investigated. Regardless of the exact mechanism behind this phenomenon, it may well be that the greater NADP-dependent 11β-HSD activity in response to high concentrations of glucocorticoids offers cells (additional) protection against elevated levels of cortisol, since this hormone will be inactivated more rapidly.

It is interesting to note from our results that in LLC-PK$_1$ cells, the NADP-dependent 11β-HSD activity is enhanced by estradiol, whereas it is known that estrogens directly repress 11β-HSD 1 mRNA in rat liver *(33)*. Further, the administration of estradiol to rats decreased the NADP-dependent 11β-HSD 1 enzyme mRNA in kidney to almost undetectable levels while markedly increasing NADP-dependent 11β-HSD activity *(33)*. The dissociation between message for renal 11β-HSD 1 and NADP-dependent activity in estradiol-treated rats has been interpreted as a product from a different gene *(34)*.

Using an antibody raised against mouse liver 11β-HSD 1, we failed to detect immunoreactive protein in Western blots of (untreated as well as steroid-treated) LLC-PK$_1$ homogenates, even when a sensitive chemiluminescent detection method was applied. Moreover, in RT-PCR experiments, we did not detect any 11β-HSD 1 mRNA signal in these cells when applying primers homologous to conserved regions of both mouse and human 11β-HSD 1 cDNA.

It is possible that LLC-PK$_1$ cells contain, other than 11β-HSD 1 and in addition to 11β-HSD 2, a distinct kinetic isoform that is responsible for the occurrence of the NADP-dependent 11β-HSD oxidative activity described in our investigations. Additional 11β-HSD isoforms have already been mooted *(34–38)*. Recently, a new high-affinity NADP-dependent 11β-HSD isoform with exclusively oxidase activity was reported to be present in the choriocarcinoma cell line JEG-3 *(36)*. In guinea pig liver, an NAD-dependent third 11β-HSD isozyme has been suggested *(37)*. The existence of a third, unidirectional NADP-dependent enzyme, 11β-HSD 3, has been proposed in sheep kidney *(34)* and rat Leydig cells *(38)*. Additional 11β-HSD genes may indeed exist, since from some structurally related short-chain dehydrogenases/reductases (17β-HSD and 3β-HSD) it is known that these are each encoded by multiple genes.

In conclusion, our results are indicative of the absence of 11β-HSD 1 in the renal cell line LLC-PK$_1$, a fact that has

also been suggested by other laboratories *(20,39)*. Rather, in addition to 11β-HSD type 2, our observations point to the existence of a third 11β-HSD isoform in LLC-PK$_1$ cells, which shares some (but not all) features with 11β-HSD 1. Clearly, the (unambiguous) indentification of this NADP-dependent 11β-HSD isoform in LLC-PK$_1$ cells requires further study.

Materials and Methods

Chemicals and Materials

Except cortisol (ultrapure grade from Fluka, Switzerland) and the glucocorticoid antagonist RU486 (mifepristone; Biomol, Hamburg, Germany), steroids were obtained from Sigma (St. Louis, MO). Cosubstrates were from Boehringer Mannheim (Germany). Sterile plasticware was obtained from Costar (High Wycombe, Buchs, UK), Medium 199, FCS, and antibiotics were from Gibco (Eggenstein, Germany). All other chemicals, of analytical or higher grade, were from Merck (Darmstadt, Germany).

Cell Culture and Harvesting

LLC-PK$_1$ cells, kindly provided by Prof. Dr. Rosenthal (Rudolf-Buchheim Institute of Pharmacology, University of Giessen, Germany), were maintained under 5% CO_2 at 37°C in Medium 199 with Earle's salts, supplemented with 10% FCS, 50 IU/mL of penicillin, and 50 μg/mL of streptomycin. Medium was exchanged every 3 d. Cells were plated out in Petri dishes at 500–750 cells/mm^2, grown to confluence, and replated (after treatment with 0.05–0.02% trypsin-EDTA, washing, and centrifugation) or harvested. Harvesting was conducted by removing medium; washing the cells twice with 0.25 *M* sucrose in 10 m*M* sodium phosphate buffer with 1 m*M* EDTA, pH 7.4; and scraping them off with a rubber policeman. After centrifugation (400*g*), cells were resuspended in the same buffer without sucrose (to about 50 × 10^6 cells/mL) and homogenized by sonification. By this procedure, all cells were disrupted as judged by Trypan blue exclusion. Protein content was determined according to ref. *40*.

Determination of 11β-HSD Activity

Prewarmed (2 min at 37°C) cellular homogenates (0.10–0.40 mg of protein) were added to a prewarmed mixture with cosubstrate and substrate (at 1 m*M* and 1 μ*M* final concentrations, respectively) to obtain a total incubation volume of 100 μL of 10 m*M* phosphate buffer with 1 m*M* EDTA, pH 7.4. For 11β-oxidation, cortisol was used as substrate with NAD or NADP as cosubstrates. For 11-ketone reduction, cortisone together with NADH or NADPH, was used. After 1 h of incubation at 37°C, the reaction was stopped with 50 μL of 0.2 *M o*-phosphoric acid, and steroids were extracted with methylene chloride and analyzed on HPLC as described previously *(41)*, except that an aqueous solution of 26% acetonitrile (HPLC grade) was used as mobile phase. Enzyme kinetics were determined from S-V plots by direct fitting of the Michaelis-Menten equation using the GPAD program (GraphPAD). Corticosteroid metabolism in whole cells was determined by extracting 1 mL of acidified culture medium with 1 mL of methylene chloride and subsequent analysis on HPLC.

Modulation Experiments with Steroids

Cell culture conditions were similar as previously described, except that steroids (400 μ*M* in culture medium) were added to the cells at confluence, giving the desired final concentrations (1 μ*M* for the steroid comparison experiment, 1 and 10 μ*M* for experiments with RU486, and 5–5000 n*M* for the dexamethasone dose-effect curve). Cells were harvested after 19 hours of exposure to the steroids (or 1 min to 32 h in the time-dependency experiment). Control experiments were run in parallel (adding vehicle to cells), providing similar culture times for all cells.

Statistical Analysis

Effects of treatments of cells were tested for significance by the use of one-way ANOVA and calculation of the least significant differences for α 0.05.

Immunoblotting of 11β-HSD 1

Immunization and preparation of antisera against 11β-HSD 1 of mouse liver was performed as described elsewhere *(42)*. Equal amounts of protein (30 μg) from LLC-PK$_1$ cell homogenates were submited to sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% polyacrylamide), and the separated proteins were transferred to nitrocellulose or nylon sheets. Nonspecific binding sites were blocked overnight with TBS containing Tween-20 (0.1%) and milk powder (5%). The membrane was then washed with TBS/Tween and incubated overnight with the 11β-HSD 1 antibodies (1:1000 dilution). Antigen-antibody complexes were detected by peroxidase-conjugated secondary antibodies specific for rabbit IgG (dilution 1:1000). Signals were developed either by the chloronaphthol method or by using the enhanced chemiluminescence kit (Amersham). Microsomes (10 μg of protein) from mouse liver served as positive control.

RT-PCR Studies

RT-PCRs of 11β-HSD 1 and 11β-HSD 2 in LLC-PK$_1$ cells were performed according to standard protocols by using Ready-To-Go™ RT-PCR Beads from Amersham Pharmacia Biotech. In brief, RNA was isolated from LLC-PK$_1$ cells, or mouse and human liver by the RNeasy Kit (Qiagen, Hilden) as described in the manual, supplemented with RNase inhibitor RNasin (MBI) (10 vol%), and quantitated spectrophotometrically at 260 nm. RT-PCR reactions were run in a final volume of 50 μL, each containing 2.0 U of *Taq* DNA polymerase, 10 m*M* Tris-HCl, 60 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 μ*M* of each dNTP, Moloney Murine Leukemia Virus reverse transcriptase (FPLC*pure*™), RNAguard™ ribonuclease inhibitor (porcine), and RNase/DNase-free bovine serum albumin, supplemented with

RNase-free water (0.1% diethylpyrocarbonate prior to sterilization). Reverse transcription of 1 μg of RNA was performed for 30 min at 42°C and 5 min at 95°C using 20 pmol of specific forward and reverse primers. PCR conditions for 11β-HSD 1 were as follows: 40 cycles for 1 min at 95°C, 1 min at 58°C (human) or 60°C (mouse), and 1 min at 72°C. PCR conditions for 11β-HSD 2 were 40 cycles with 30 sec at 96°C, 45 sec at 56°C, and 90 sec at 72°C. PCR reactions were completed by 10 min at 72°C. The specific primers of human 11β-HSD 1 were as follows: forward primer, 5'-GAATTCAGACCAGAGATGCTC; reverse primer, 5'-AACTGAGGAAGTTGACTTCCA *(43)*. The specific primers of mouse 11β-HSD 1 were as follows: forward primer, 5'-TTGTAAGTAACTAGGAACTCCTG; reverse primer, 5'-CATAGTTACAATCAAGTTCACAA *(9)*. The 11β-HSD 2 encoding primers used were as follows: forward primer 5'-TGCTGCAGATGGACCTGACCAA; reverse primer, 5'-TAGTAGTGGATGAAGTACATGAGC *(20)*.

Aliquots (20 μL of RT-PCR products) were then subjected to electrophoresis in 3% agarose gel and visualized by staining with ethidium bromide. All results were reproduced in three separate experiments.

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