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11β-Hydroxysteroid Dehydrogenase Type 2 Complementary Deoxyribonucleic Acid Stably Transfected into Chinese Hamster Ovary Cells: Specific Inhibition by 11α-Hydroxyprogesterone*

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ABSTRACT

The 11β-hydroxysteroid dehydrogenase type 2 (11βHSD-2) enzyme is thought to confer aldosterone specificity upon mineralocorticoid target tissues by protecting the mineralocorticoid receptor from binding by the more abundant glucocorticoids, corticosterone and cortisol. We have developed a Chinese hamster ovary cell line stably transfected with a plasmid containing the rat 11βHSD-2 complementary DNA. This cell line has expressed the enzyme consistently for many generations. The 11βHSD-2 was located primarily in the microsomes, but significant amounts also existed in the nuclei and mitochondria. The enzymatic reaction was unidirectional, oxidative, and inhibited by the product, 11-dehydrocorticosterone, with an IC_{50} of approximately 200 nM. The K_m for corticosterone was 9.6 ± 3.1 nM, and that for NAD^+ was approximately 8 μM. The enzyme did not convert dexamethasone to 11-dehydrodexamethasone. Tunicamycin, an *N*-glycosylation inhibitor, had no effect on enzyme activity. 11α-Hydroxyprogesterone (11αOH-P) was an order of magnitude more potent a competitive inhibitor of the 11βHSD-2 than was glycyrrhetinic acid (GA) (approximate IC_{50} = 0.9 *vs.* 15 nM). 11βOH-P, progesterone, and GA were almost equipotent (IC_{50} = 10 and 6 nM, respectively), and 5α-pregnandione and 5β-pregnandione were less potent (IC_{50} = 100 and 500 nM, respectively) inhibitors of the enzyme.

When the inhibitory activities were examined with intact transfected cells, 11αOH-P was more potent than GA (IC_{50} = 5 and 150 nM, respectively). 11αOH-P was not metabolized by 11βHSD-2. We were unable to demonstrate the presence of 11αOH-P in human urine.

In conclusion, a cell line stably transfected with the rat 11βHSD-2 was created, and the enzyme kinetics, including inhibition, were characterized. 11αOH-P was found to be a potent relatively specific inhibitor of the 11βHSD-2 enzyme. Its potential importance is that it is the most specific inhibitor of the 11βHSD-2 so far encountered and would aid in the study of the physiological importance of the isoenzyme. (*Endocrinology* **137:** 2308–2314, 1996)

ALDOSTERONE acts through the mineralocorticoid receptor (MR) in the kidney to produce sodium retention and potassium excretion, whereas corticosterone (B) and cortisol, although 100-1000 times as abundant as aldosterone, normally produce very few renal mineralocorticoid effects. The 11β-hydroxysteroid dehydrogenase (11βHSD) enzymes inactivate B and cortisol by converting them to 11-dehydrocorticosterone (A) and cortisone. The MR has similar affinity for aldosterone, B, and cortisol (1–4). Rather than having intrinsic properties that confer binding specificity, selectivity for aldosterone is conferred by the presence of 11βHSD activity in aldosterone target tissues. Oxidation of cortisol and B allows aldosterone, which is not metabolized by 11βHSD, access to the receptor (5–7). 11βHSD activity has also been shown to modulate glucocorticoid binding to the glucocorticoid receptor (8, 9). This is particularly crucial in the placenta, where 11βHSD maintains normal fetal blood levels in the face of elevated maternal glucocorticoids (10), and in the hypothalamus, where it modulates access of cortisol and B to the hypothalamo-pituitary-adrenal feedback system (9).

Two 11β-hydroxysteroid dehydrogenases have been cloned (11–13). Assignment of the title of MR gatekeeper to the first 11βHSD isolated provided an imperfect answer to the question of MR specificity. 11βHSD-1 is $NADP^+$ dependent, has a high K_m for cortisol and B (1–3 μM) that is above the physiological range of circulating free glucocorticoids (~10 nM), does not colocalize with the MR in the kidney, and exhibits bidirectional activity (14, 15). 11βHSD-2, more recently described, is NAD^+ dependent, has a K_m of 4–14 nM (closer to the range that would be relevant to physiological amounts of glucocorticoids), and colocates with the MR in the kidney (13, 16). It has been cloned from human (13), sheep (12), rabbit (17), and rat (18) complementary DNA (cDNA) libraries.

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Glycyrrhetinic acid (GA; the principal constituent of licorice) and carbenoxolone (CBX; a hemisuccinate derivative of GA) are inhibitors of 11βHSDs, cause sodium retention and hypertension, and have been used to study the role of the enzyme(s) in mineralocorticoid action (6, 7, 19). GA-like factors (GALFs), which inhibit 11βHSD and steroid 5β-reductase activity, were found in extracts of human urine, and their excretion was increased in pregnancy and congestive heart failure (20, 21). Recently, it was reported that 11α- and 11β-hydroxyprogesterone (11αOH-P and 11βOH-P) were potent inhibitors of 11βHSD-2 both *in vitro* and *in vivo* and may be important endogenous inhibitors of the enzyme (22).

We recently cloned a rat cDNA and demonstrated that modified Chinese hamster ovary (CHO) cells transiently transfected with the rat 11βHSD-2 cDNA convert B to A (18). The level of enzyme expression in transiently transfected cell lines, however, is variable, and the kinetic properties of the enzyme are difficult to establish with consistency. We have established a stably transfected CHO cell line that consistently expresses 11βHSD-2 and provided enough enzyme for characterization. We now report studies of the kinetics of the 11βHSD-2 and quantitative effects of exogenous and possibly endogenous inhibitors on the enzyme activity *in vitro*.

Materials and Methods

Materials

Steroids, CBX, GA, tunicamycin, NAD^+, and $NADH^+$ were purchased from Sigma Chemical Co. (St. Louis, MO). Reagent grade solvents were obtained from Fisher Scientific (Medford, MA). Lipofectamine and geneticin were obtained from Life Technologies (Grand Island, NY). [1,2-^{3}H]B (SA, 25 Ci/mmol) and [1,2,4,6,7-^{3}H]dexamethasone (SA, 70 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). [1,2-^{3}H]A was synthesized from [1,2-^{3}H]corticosterone-21-acetate by oxidation with Jones reagent and hydrolysis with potassium bicarbonate. Channeled TLC plates (silica gel 60 A) were purchased from Whatman (Clifton, NJ). RPMI 1640 and fetal clone II serum (FCII) were supplied by the campus core resource and HyClone Laboratories (Logan, UT), respectively. The CHO-K1 cell line was obtained from American Type Culture Collection (Rockville, MD) and was chosen for stable transfection because it has no detectable intrinsic 11βHSD-2 activity.

Stable transfection of rat 11βHSD-2 cDNA into a CHO cell line

The plasmid pcDNA3/neo-11βHSD-2, which contains the entire coding region of the rat 11βHSD-2, was prepared as described previously (18).

CHO cells were grown in RPMI 1640 supplemented with 10% FCII in a T-75 culture flask at 37 C to about 70% confluence. The cells were washed once with serum-free RPMI 1640 and incubated with 8 ml serum-free RPMI 1640 premixed with 6 μg plasmid DNA and 48 μl lipofectamine for 5 h at 37 C. Additional medium (8 ml) containing 20% FCII was added, and the culture was continued for 48 h. The cells were then transferred to five 96-well culture plates and cultured with medium containing 10% FCII and 300 μg/ml geneticin for 3 weeks, with medium changes every 3 days, until the surviving clones were large enough to be seen easily. Medium containing 10 ng/ml B was added to wells containing clones, incubated overnight, and collected for assay of A by enzyme-linked immunosorbent assay (ELISA) to identify the clones expressing the cDNA. The positive clones were amplified, and those with the highest enzymatic activity were used for the studies.

Cells expressing the highest enzymatic activity were cloned by limiting dilution in 96-well plates by plating at concentrations of 0.5, 1, and 10 cells/well. Positive wells at the lowest dilution were expanded, and aliquots were frozen in liquid nitrogen.

Preparation of nuclei, mitochondria, microsomes, and cytosols from stably transfected CHO cells

Stably transfected CHO cells were cultured in several 225-cm^2 flasks until confluent, then scraped and washed. The cells were resuspended in ice-cold homogenizing buffer (50 mM Tris-HCl, 1 mM $MgCl_2$, and 0.25 M sucrose, pH 8.0) and homogenized in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Organelles were isolated by differential centrifugations. Briefly, the lysate was centrifuged at 680 × *g* for 10 min at 4 C for a pellet containing the nuclei. The supernatant was then centrifuged at 9,000 × *g* for 10 min to obtain the mitochondrial fraction. This supernatant was centrifuged at 100,000 × *g* for 60 min to obtain the microsomes, and the resultant supernatant was used as the cytosolic fraction. Pellets were reconstituted in incubation buffer (50 mM Tris-HCl and 1 mM $MgCl_2$, pH 8.0). Nuclei were further purified according to a two-step procedure (23). In short, the crude nuclei fraction (nuclei-1) was centrifuged at 70,000 × *g* for 70 min using a discontinuous sucrose gradient of 0.25/2.4 M sucrose in 10 mM HEPES (pH 7.5; nuclei-2), and the pellet was centrifuged over a 2.2-M sucrose cushion in 10 mM HEPES (pH 7.5) at 70,000 × *g* for 60 min (nuclei-3). Microsomal contamination was estimated by measuring the microsomal marker glucose-6-phosphatase (24). Subcellular distribution was examined immediately after all fractions were obtained. Protein concentrations were determined by a BCA protein assay kit (Pierce Chemical Co., Rockford, IL). Extra microsomes were frozen at −80 C in 50% glycerol for future use in other studies with no significant loss of activity.

Measurement of enzymatic activity

Five micrograms of protein were incubated for 15 min at 37 C with 200,000 dpm [1,2-^{3}H]B in 500 μl of the incubation buffer containing 0.5 mM NAD^+. To determine whether dexamethasone is oxidized, [1,2,4,6,7-^{3}H]dexamethasone was similarly incubated, and conversion to the 11-keto derivative wasmeasured. Reductase activity was measured by incubation with 200,000 dpm [1,2-^{3}H]A in a buffer containing 0.5 mM NADH. The incubations were terminated by the addition of 5 ml methylene chloride, 20 μg unlabeled B and A or unlabeled dexamethasone and 11-dehydrodexamethasone were added as markers, and the steroids were extracted. Extracts were concentrated, and the steroids were separated by TLC in acetone-methylene chloride (18:82, vol/vol). Areas corresponding to the steroids were located under UV light, scraped, eluted with 500 μl isopropanol, and counted by scintillation spectrometry. All experiments were performed in triplicate and included tritium steroid/buffer blanks.

Subcellular distribution of 11βHSD-2

Five micrograms of protein from each fraction were incubated for 15 min at 37 C with 200,000 dpm [1,2-^{3}H]B in 500 μl incubation buffer containing 0.5 mM NAD^+ and processed as described above.

Inhibition of 11βHSD-2 activity by licorice derivatives and other steroids

Microsomes (5 μg) were incubated for 15 min in 500 μl incubation buffer containing 0.5 mM NAD^+ with 100,000 dpm [1,2-^{3}H]B with serial dilutions of 1 nM to 1 μM B, progesterone, 5α-dihydroxyprogesterone, 5β-dihydroprogesterone, 11β-hydroandrostenedione, 11βOH-P, CBX, GA, and 10 pM to 1 μM 11αOH-P.

Inhibition of 11βHSD-2 activity by B, GA, and 11αOH-P in intact stably transfected CHO cells

Cells were grown in 24-well plates until confluent. The medium was substituted with 0.5 ml RPMI 1640 containing 200,000 dpm [1,2-^{3}H]B with 1 nM to 3 μM B or GA, or 10 pM to 100 nM 11αOH-P, and the cells were incubated for 15 min.

Effect of tunicamycin on 11βHSD-2 activity in intact cells

11βHSD-2 has a potential glycosylation site (18). To determine whether glycosylation was important for activity, cells were preincubated with 1 ng/ml to 1 μg/ml tunicamycin for 24 h (25). They were then incubated for 60 min with 0.5 ml RPMI 1640 containing 200,000 dpm [1,2-^{3}H]B and the same concentrations of tunicamycin. The supernatant was processed as described above.

Metabolism of 11αOH-P

To explore the possibility that 11αOH-P is also a substrate for 11βHSD-2, 1 mg microsomes from the transfected cells was incubated in 20 ml buffer containing 0.5 mM NAD^+ and 20 nM 11αOH-P for 1 h at 37 C. At the end of the incubation, the buffer was extracted with dichloromethane, washed, and evaporated. The extract was dissolved in acetonitrile and subjected to HPLC quantification of 11αOH-P and the putative product 11-ketoprogesterone. The system used was with a Hypersil ODS 5-μm column (Alltech Associates, Inc., Deerfield, IL) eluted with an isocratic system of acetonitrile-water (35:65, vol/vol).

Excretion of 11αOH-P in human urine

Ten milliliters of urine from a normal subject (male), a pregnant woman, and a patient with glucocorticoid-suppressible aldosteronism were extracted with 10% dichloromethane in hexane and subjected to HPLC as described above. The eluate was followed by UV detection at 240 nm and collected in 1-ml fractions. The fractions were evaporated, reconstituted in an ELISA buffer (PBS, 0.5% BSA, and 0.05% Tween 20), and subjected to ELISA using a monoclonal antibody against 11αOH-P hemisuccinate (26). This antibody has a sensitivity of about 5 pg/well.

Inhibition of 11αOH-P and GA of the $NADP^+$-dependent dehydrogenase and NADPH oxidoreductase activities in rat kidney

Rat kidney microsomes (100 μg) were prepared as described above and incubated with 200,000 dpm [1,2-^{3}H]B with serial dilutions of 0.1 μM to 100 μM B, GA, and 11α-OH-P in the presence of 0.5 mM $NADP^+$ for 15 min at 37 C. To measure the reductase activity, they were also incubated with 200,000 dpm [1,2-^{3}H]A with serial dilutions of 0.1 μM to 100 μM A or 11αOH-P in the presence of 0.5 mM NADPH for 15 min at 37 C. The conversion was measured as described above.

Statistical analysis

The K_m of the enzyme for B and NAD^+ was obtained using Lineweaver-Burk plots (double reciprocal plots). The IC_{50} values of inhibitors for the enzyme were calculated from a dose-response curve in comparison to either B (dehydrogenation) or A (oxido-reductase). Lineweaver-Burk plots were also used to determine whether the inhibitor was competitive or noncompetitive. All data were expressed as the mean ± SD.

Results

Subcellular distribution

The highest enzymatic activity was found in the microsomal fraction (14.7 ± 0.7 pmol/min·μg protein), as shown in Fig. 1. Significant activity was also observed in the crude nuclear (nuclei-1; 8.7 ± 0.1 pmol/min·μg protein) and mitochondrial (6 ± 0.9 pmol/min·μg protein) fractions, and very low activity was present in the cytosol (0.4 ± 0.1 pmol/min·μg protein). To decrease the degree of microsomal contamination in the nuclear fraction, nuclei were purified as described above. The partially purified nuclei (nuclei-2 in Fig. 1) had an activity of 7.8 pmol/μg protein. Further purification decreased the activity to 3.5 ± 0.2 pmol/μg protein in the nuclei-3 fraction. The microsomal marker glucose-6-phosphatase activity was 142 pmol phosphate liberated/μg protein in the microsomal fraction, 3.6 pmol/μg protein in the nuclei-1 fraction, 1.2 pmol/μg in the nuclei-2 fraction, and undetectable in the nuclei-3 fraction. Glucose-6-phosphatase activity was also undetectable in the mitochondrial fraction and cytosol.

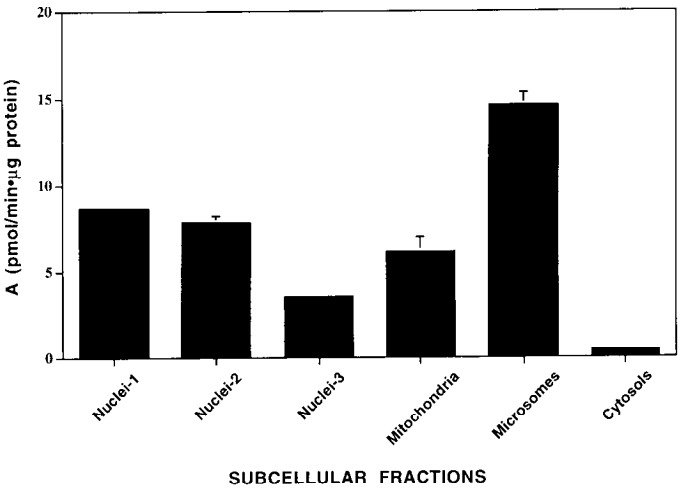


FIG. 1. Subcellular distribution of rat 11βHSD-2 in cells transfected with rat 11βHSD-2 plasmid cDNA into CHO cells. Subcellular organelles were prepared by differential centrifugation. Crude nuclei (nuclei-1) were further purified by centrifugation over a discontinuous gradient of 0.25/2.4 M sucrose (nuclei-2), followed by centrifugation over a 2.2-M sucrose cushion (nuclei-3). Five micrograms of organelle protein were assayed for 11HSD-2 activity for 15 min at 37 C. The results are presented as picomoles of A formed per μg protein (mean ± SD).

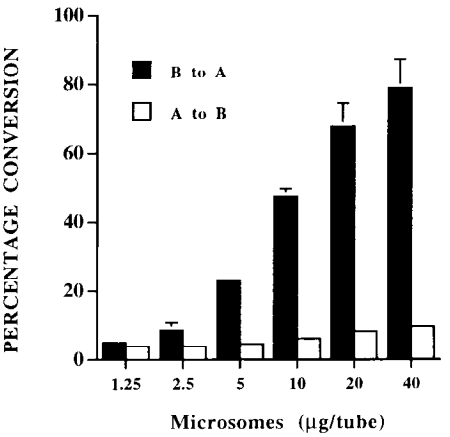


FIG. 2. Determination of dehydrogenase and oxido-reductase activities of rat 11βHSD-2 measured using different concentrations of microsomal protein. Cells were incubated with 0.5 mM NAD^+ or NADH for dehydrogenase and oxido-reductase reactions, respectively. Results are presented as the mean percent conversion (±SD) of [^{3}H]B to [^{3}H]A (dehydrogenase) or of [^{3}H]A to [^{3}H]B (oxido-reductase).

Measurement of enzymatic activity

The enzymatic activities after the addition of either NAD^+ plus [1,2-^{3}H]B or NADH plus [1,2-^{3}H]A in relation to microsomal protein concentration are shown in Fig. 2. The

dehydrogenase reaction increased in a protein-dependent manner. The reductase activity was much lower than the dehydrogenase reaction, and only a small amount of conversion was observed at higher concentrations of protein. These studies indicate that the reaction is almost exclusively unidirectional.

The K_m for B conversion to A by the microsomes was 9.6 ± 3.1 nM (n = 7). This value is similar to that reported using homogenates of transiently transfected modified CHO cells (10.2 ± 2.2 nM) (18). The K_m for the cofactor NAD^+ was 8 μM.

Conversion of dexamethasone to 11-dehydrodexamethasone

The enzyme in the stably transfected cells did not metabolize [1,2-^{3}H]dexamethasone to [1,2-^{3}H]11-dehydrodexamethasone (1.4 ± 0.01% conversion, which was not significantly different from the blanks). In simultaneous control incubations, [1,2-^{3}H]B was efficiently metabolized to [1,2-^{3}H]A (46.5 ± 2.9% conversion).

Product inhibition

Transfected cell microsomes were incubated with [1,2-^{3}H]B and increasing concentrations of unlabeled A. The conversion of [1,2-^{3}H]B to [1,2-^{3}H]A is shown in Fig. 3. The reaction was inhibited by A, the product, with an IC_{50} of approximately 200 nM.

Effect of inhibitors on microsomal enzyme

Log dose-response curves of eight different steroid inhibitors of 11HSD-2 enzymatic activity are shown in Figs. 4 and 5. Progesterone was slightly more potent than B (IC_{50} = 6 *vs.* 10 nM). The IC_{50} values for the progesterone metabolites, 5α-dihydroprogesterone and 5β-dihydroprogesterone, were 100 and 500 nM, respectively. The IC_{50} for 11βOH-P was 10 nM. 11αOH-P was the most potent inhibitor, with an IC_{50} of 0.9 nM, which is more than 10 times as potent as GA (IC_{50} = 15 nM) or CBX (IC_{50} = 10 nM). The Lineweaver-Burk plot (Fig. 6) of 11αOH-P and GA inhibition of the conversion of [1,2-^{3}H]B to [1,2-^{3}H]A indicates that they are competitive inhibitors.

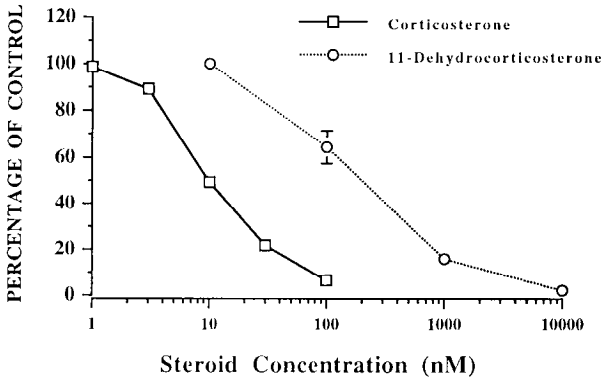


FIG. 3. End product inhibition. Microsomes (8 μg) were incubated with increasing concentrations of B or A and 200,000 dpm [^{3}H]B. The conversion of [^{3}H]B to [^{3}H]A was measured by TLC, and results are presented as a percentage of control conversion (±SD).

Effects of B, 11αOH-P, and GA on the conversion of [1,2-^{3}H]B to [1,2-^{3}H]A in intact stably transfected cells

The inhibition was also measured in intact cells, which present a membrane barrier for access of the steroids to the intracellular enzyme. The inhibition of [1,2-^{3}H]B conversion to [1,2-^{3}H]A in cells incubated with log increasing doses of unlabeled B, 11αOH-P, and GA is shown in Fig. 6. 11αOH-P was the most potent inhibitor of the conversion, with an apparent IC_{50} of 5 nM, about one fifth its potency in the microsomal preparation. IC_{50} values for B and GA were approximately 150 and 500 nM, respectively, 15 and 33 times lower than their IC_{50} values in microsomal preparations.

Effect of tunicamycin on enzyme activity

Inhibition of glycosylation with concentrations of up to 1 μg/ml tunicamycin did not inhibit 11βHSD-2 activity.

Metabolism of 11αOH-P

Microsomes incubated with 11αOH-P showed no modification of the steroid, as estimated by sensitive HPLC measurements.

Excretion of 11αOH-P in human urine

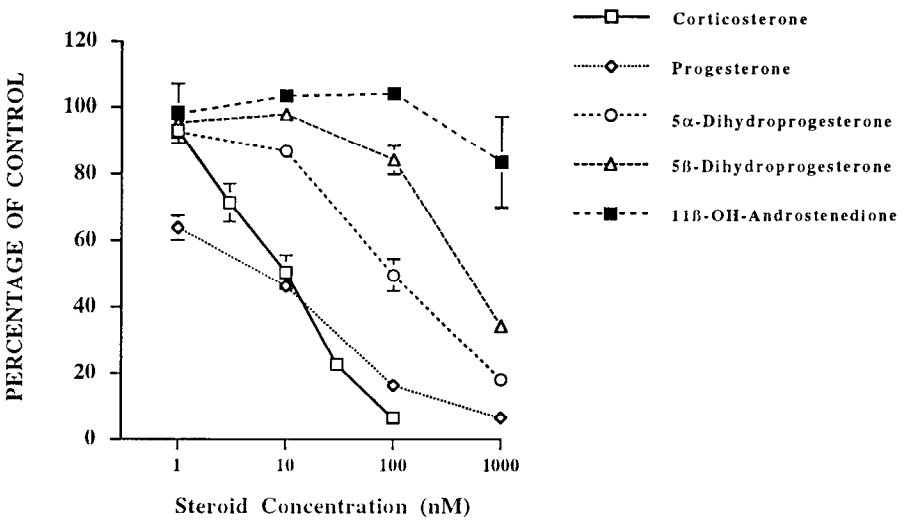
As 11αOH-P is a potential metabolite of progesterone, we investigated the possible presence of the steroid as an endogenous GALF (20), but were unable to demonstrate its presence in the urine of normal men, pregnant hypertensive women, or a single patient with glucocorticoid-suppressible hypertension using a very sensitive HPLC separation followed by ELISA.

Effects of 11αOH-P and GA on the conversion of [^{3}H]B to [^{3}H]A and [^{3}H]A to [^{3}H]B in rat kidney microsomes

Rat kidney microsomes containing primarily the 11βHSD-1 isoenzyme converted [^{3}H]B to [^{3}H]A with a K_m of 1.5 μM, as described by others (27). The IC_{50} of GA was approximately 80 nM; that of 11αOH-P was 0.7 μM. [^{3}H]A to [^{3}H]B conversion was inhibited by A and 11αOH-P with IC_{50} values of 3 and 10 μM, respectively. These results are similar to those reported previously (22).

Discussion

The enzyme 11βHSD-2 is found in mineralocorticoid target organs, including the distal tubules of the kidney, salivary glands, distal colon, and adrenal, and in select areas of the brain (18, 28). Study of the enzymatic characteristics of 11βHSD-2 has been difficult because of its restricted distribution and coexpression with 11βHSD-1 in several tissues, including the kidney (29). The CHO cell line does not have innate 11βHSD activity, but when stably transfected with the rat 11βHSD-2 cDNA, it expressed significant 11βHSD-2 activity. The enzyme is located primarily in the microsomes and is unidirectional and NAD^+ dependent. The apparent K_m values for the 11βHSD-2 enzyme for B and NAD^+ are 9.6 nM and 8 μM, respectively. These results are consistent with



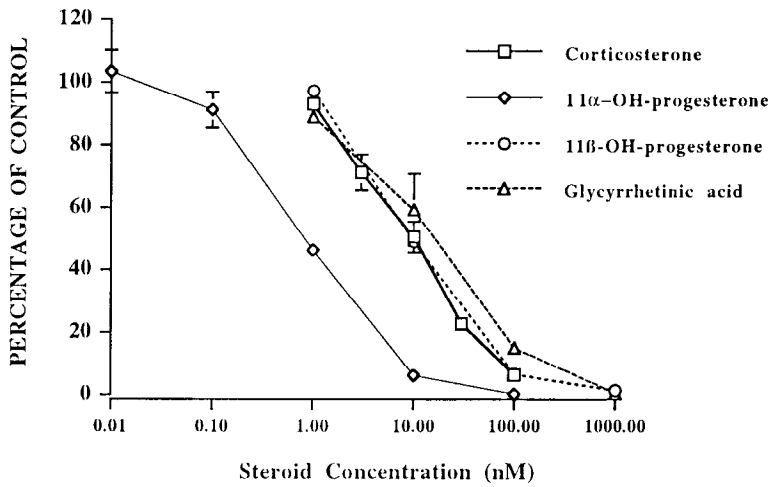


FIG. 4. Inhibition of microsomal 11HSD-2 activity by steroids and licorice derivatives. Eight micrograms of microsomal protein were incubated with increasing concentrations of the various steroids, and the conversion of [^{3}H]B to [^{3}H]A was measured by TLC. Results are presented as a percentage of control conversion (±SD).

those of our previous report using transiently transfected cells (18). The enzyme exhibits dose-dependent product inhibition; the IC_{50} of A for the enzyme was approximately 200 nM. Product inhibition is a characteristic of 11βHSD-2, but not of 11βHSD-1 (12). Dexamethasone was reported to be a substrate for the human 11βHSD-2; however, the kinetic characteristics of the metabolism of dexamethasone by human 11βHSD-2 have not been reported. It is possible that a statistically significant metabolism may be demonstrated that is too low to be biologically insignificant. In our studies, the conversion of dexamethasone to 11-dehydrodexamethasone by cells transfected with rat 11βHSD-2 was not statistically different from that in our blanks.

N-Glycosylation occurs by the transfer of core oligosaccharide by the enzyme oligosaccharyl transferase to the asparagine residue of a conserved tripeptide sequence, Asn-X-Ser/Thr. Dehydrogenase activity of rat 11βHSD-1, in which there are two such potential glycosylation sites, decreased in transfected cells grown in the presence of tunicamycin (25). Rat 11βHSD-2 has a potential glycosylation site at residues 392–394. The 11βHSD-2 activity of transfected cells incubated with tunicamycin was unchanged, suggesting that either glycosylation is not important for full expression of the enzymatic activity or the potential site is not glycosylated.

Since Semafuko *et al.* (20) reported the existence of GALFs in human urine, especially that of pregnant individuals and those in congestive heart failure, several substances have been studied as possible endogenous inhibitors of 11βHSD activity, including bile acids (30) and progestagens (31). Murphy (32) showed that at term, fetal blood contained high concentrations of inhibitors of placental 11βHSD, and these inhibitors were probably 11β-hydroxymetabolites of progesterone, specifically 11βOH-P and 11β-hydroxypregnenolone. Recently, Souness *et al.* (22) demonstrated that 11αOH-P and 11βOH-P are potent inhibitors of 11βHSD-2 both *in vitro* and *in vivo* and suggested their possible role as endogenous 11βHSD inhibitors. Both steroids conferred mineralocorticoid activity upon B in adrenalectomized animals at doses substantially lower than those required for CBX or GA to exert a similar effect (20 μg *vs.* 2.5 mg). We demonstrated that 11αOH-P inhibition of rat 11βHSD-2 was 15 and 100 times more potent than that of GA in microsomes (0.9 *vs.* 15 nM) and intact cells (5 *vs.* 500 nM), respectively. Our

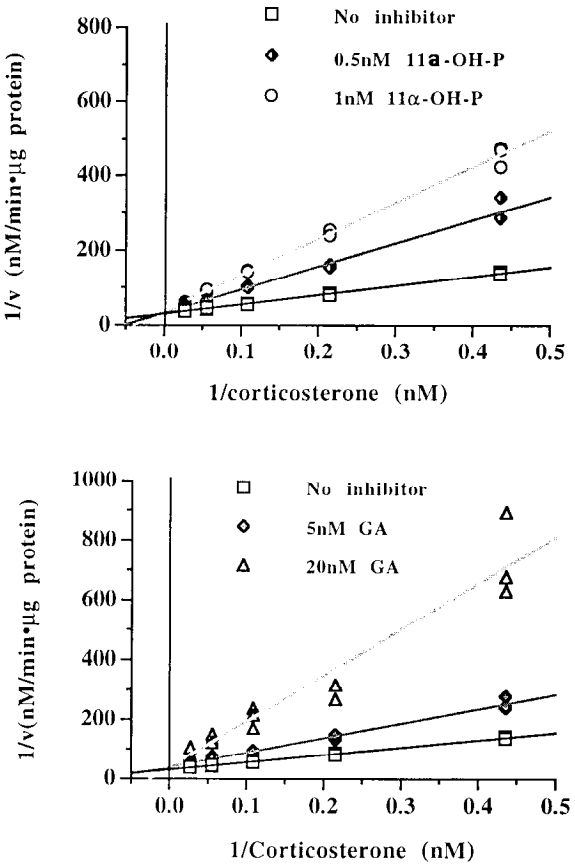


FIG. 5. Lineweaver-Burk plots of inhibition of 11HSD-2 activity by 11αOH-P and GA. Microsomes (8 μg) were incubated with increasing concentrations of [^{3}H]B with and without 0.5 and 1 nM 11αOH-P or 5 and 20 nM GA. The conversion of [^{3}H]B to [^{3}H]A was measured by TLC, and the results are presented as a double reciprocal plot.

IC_{50} values for 11βHSD inhibition by 11αOH-P were significantly lower than those reported by Souness *et al.* (22); however, these differences may be due to different enzyme sources, as they used lysates of a human choriocarcinoma cell line, JEG-3 (22). Monder *et al.* (33) reported that the K_i of GA with rat kidney microsomes ranged from about 10^{-9}-10^{-8} M, whereas that of intact tubules was about 10^{-6}-10^{-5} M. They concluded that intact tissues had a low permeability to GA. The reported IC_{50} for GA in microsomes was similar to the value we found. The difference in inhibitory activity between 11αOH-P and GA was larger in intact cells than in microsomes, suggesting that 11αOH-P is more permeable to the cells than GA and partially explains the greater bioactivity of 11αOH-P compared to that of GA in conferring mineralocorticoid activity to B in the intact animal (22). Accordingly, 20 μg 11αOH-P, 500 μg 11βOH-P, and 2.5 mg CBX administered with 100 μg B had similar bioactivities (natriuresis and kaliuresis) in adrenalectomized rats (22). The inhibition of 11-oxido-reductase by 11αOH-P was significantly lower than that by GA, indicating that 11αOH-P is a more specific inhibitor of 11βHSD-2 than GA.

Progesterone, which is not hydroxylated at the 11 position, also inhibited 11βHSD-2 with a potency similar to that of B. Progesterone is a potent ligand for the mineralocorticoid receptor, with an affinity even higher than that of aldosterone, but it has only antagonistic properties (34). The biological significance of progesterone as an inhibitor of 11βHSD-2 is unclear, but it is unlikely that it acts as a GALF, conferring mineralocorticoid properties to B, because it has concurrent antagonistic properties at the MR level.

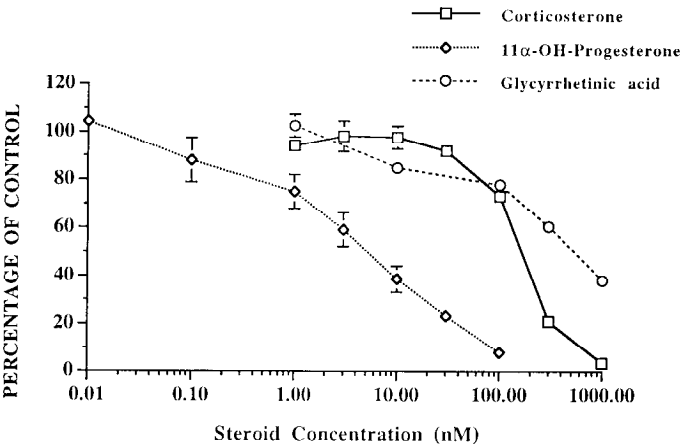


FIG. 6. Inhibition of 11HSD-2 activity in intact transfected cells by 11αOH-P and GA. Cells were grown in 24-well plates and incubated with [^{3}H]B and increasing concentrations of unlabeled B, 11αOH-P, and GA. The conversion of [^{3}H]B to [^{3}H]A was measured by TLC. Results are presented as a percentage of control conversion (±SD).

11αOH-P is a competitive inhibitor of rat 11βHSD-2, suggesting that 11αOH-P may serve as a substrate of the enzyme. However, neither the synthesis of 11αOH-P nor the existence of an 11α-hydroxylase required for its synthesis, has been demonstrated in mammalian tissues or urine. We were unable to demonstrate metabolic conversion of 11αOH-P into the putative 11-ketoprogesterone even when a very large concentration of microsomes was incubated for a relatively long period of time, nor could we demonstrate the excretion of 11αOH-P in humans using a combination of HPLC and ELISA.

These studies indicate that 11αOH-P is a more potent and specific inhibitor of the 11βHSD-2 enzyme and penetrates cells more easily than the licorice derivatives GA or CBX, which is concordant with the report that 11αOH-P has significantly greater ability to confer mineralocorticoid properties upon B (22). Although it almost certainly is not pathophysiologically important, 11αOH-P could be a useful tool for study of the role of the 11βHSD-2 enzyme in the physiology of mineralocorticoid or glucocorticoid action.

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