Selective inhibition of human type 1 11β-hydroxysteroid dehydrogenase by synthetic steroids and xenobiotics

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Abstract Functional analyses were performed with microsomal human 11β-hydroxysteroid dehydrogenase type 1 overexpressed in the yeast *Pichia pastoris*. Cell extracts or microsomes from transformed strains displayed dehydrogenase and reductase activities, which were up to 10 times higher than in human liver microsomes, while for whole cells cortisone reduction but no dehydrogenase activity was observed. The synthetic glucocorticoids prednisolone and prednisone were efficiently metabolized by subcellular fractions, whereas no activity was observed with dexamethasone, budesonide and deflazacort. Inhibitors found to be effective towards the recombinant 11β-hydroxysteroid dehydrogenase include synthetic steroids and xenobiotic compounds, revealing selective inhibition of the reaction direction, useful for development of specific inhibitors.

Key words: 11β-Hydroxysteroid dehydrogenase; Glucocorticoid; Short-chain dehydrogenase/reductase; Yeast expression; Membrane protein

1. Introduction

Glucocorticoid (GC) hormone action is achieved by specific intracellular hormone receptors acting on DNA response elements thereby regulating repression or enhancement of gene transcription [1,2]. The enzyme 11β-hydroxysteroid dehydrogenase (EC 1.1.1.146, 11β-HSD) plays an important role in this concept by catalyzing the interconversion between the GC receptor ligand (cortisol in humans) and the non-binding dehydro product (cortisone in humans). 11β-HSD therefore constitutes the major factor in a prereceptor hormone control mechanism [3,4].

Thus far, two different 11β-hydroxysteroid dehydrogenase forms have been characterized at the molecular level, although further forms are also likely to exist [4,5]. The two known isozymes belong to the short-chain dehydrogenases/reductases (SDR) superfamily [6], are microsomal enzymes, display a mutually exclusive cellular expression pattern, and differ in kinetic properties. Type 1 11β-hydroxysteroid dehydrogenase (11β-HSD-1) was first isolated from liver, but has a widespread central nervous and peripheral tissue distribution pattern. It is a bidirectional enzyme which in vivo functions mainly as a reductase, thereby converting circulating cortisone to the 'receptor active' cortisol hormone [3,4,7–9], which is essential in hepatic and adipose tissue carbohydrate metabolism [9]. The type 2 form (11β-HSD-2), however, is in vivo a unidirectional enzyme and exclusively functions as a NAD^+ dependent dehydrogenase of adrenal glucocorticoids, converting cortisol to its inactive congener, hence 'protecting' the mineralocorticoid receptor against occupancy of cortisol [10,11]. In this paper we report overexpression of 11β-HSD-1 in yeast and describe its functional and kinetic properties.

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Abbreviations: 11β-HSD-1, 11β-hydroxysteroid dehydrogenase type 1; SDR, short-chain dehydrogenases/reductases; GA, glycerrhitinic acid; CBX, carbenoxolone; GC, glucocorticoid

2. Materials and methods

2.1. Materials

Steroids, naringenin and furosemide were obtained from Sigma, deflazacort from Hoechst, 22-*R*/*S* budesonide epimers and its 11-oxo metabolites from Astra Draco.

2.2. Cloning of human 11β-HSD-1

Human 11β-hydroxysteroid dehydrogenase type 1A cDNA was obtained by RT-PCR of a total RNA preparation from a liver transplantation sample. RT-PCR was carried out using primers specific for the human type 1A sequence [8]. The resulting product was ligated into the *Sna*BI/*Not*I restriction sites of the pPIC3.5 vector (Invitrogen). The sequence of the construct obtained was verified by automated DNA sequencing.

2.3. Yeast transformation, selection and overexpression

After linearization of the plasmid DNA with endonucleases *Bgl*II or *Sac*I, yeast strains KM71 or GS115 were transformed using the spheroplasting method [12]. After homologous recombination of the 11β-HSD-1 DNA into the *Pichia* alcohol oxidase (AOX1) locus, the obtained clones were selected by phenotyping for His^+Mut^+ and His^+Mut^s transformants. Recombinant strains were confirmed by PCR of genomic DNA with gene specific primers. Positive clones for each phenotype were subjected to expression trials. To exclude intrinsic activities, mock-transformed strains (vector alone, no insert) were used as background controls. Yeast clones were grown at 30°C for 3–5 days in buffered complex medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 4×10^{-5}% biotin) containing 0.5% (v/v) methanol for induction.

2.4. Analysis of glucocorticoid metabolism in whole Pichia pastoris cells

10^6 cells of a 3 day culture were grown for 60 min in buffered complex methanol medium containing different concentrations of cortisol or cortisone, ranging from 5 to 500 μmol/l. After separation of cells, the medium was extracted twice with ethyl acetate/acetic acid (99:1, v/v), the organic phase was blown down by nitrogen, and the residue was dissolved in RP-HPLC eluent and analyzed as described below.

2.5. Preparation of subcellular fraction

Yeast subcellular fractions were prepared by mechanically disrupting the washed cells with glass beads (Sigma, 245–400 μm) in 20 mM sodium phosphate buffer, 5% glycerol, 1 mM EDTA, pH 7.0, containing 1 mM PMSF. After removal of cell debris by low speed centrifugation, an S9 fraction was prepared by centrifugation for 15 min at $9000\times g$. A final microsomal pellet was obtained by PEG 4000/NaCl precipitation or by ultracentrifugation at $100\,000\times g$ of the S9 fraction for 1 h. Microsomes were resuspended in 40 mM sodium phosphate, 1 mM EDTA, 5% glycerol, pH 7.5, to a protein concentration of 1–5 mg/ml. The liver samples were homogenized in 4-fold volumes of ice-cold buffer (40 mM Tris-HCl, pH 7.5, 1 mM EDTA,

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Table 1
Kinetic constants of recombinant human 11β-HSD-1 for 11β-dehydrogenase and 11-oxo reductase activities in whole *P. pastoris* cells, S9 fractions, and microsomes from yeast and humans

Source	11β-HSD		11-oxo reduction	
	K_m	V_{max}	K_m	V_{max}
Pichia pastoris				
Whole cells	–	0	n.d.	0.38 ± 0.1[a]
S9	9.2 ± 0.8	5.6 ± 0.2	1.9 ± 0.1	0.75 ± 0.1
Microsomes	10.3 ± 0.5	16.6 ± 0.2	2.5 ± 0.2	0.55 ± 0.2
Human liver				
Microsomes	9.5 ± 0.5	2.2 ± 0.3	2.5 ± 0.1	0.07 ± 0.03

Substrates used were cortisol and cortisone for 11β-dehydrogenase and 11-oxo reductase activities, respectively. K_m is given in μmol/l, V_{max} in nmol product formed per min per mg protein. Values are given as mean ± S.D. [a] V_{max} given as nmol product per hour per 10^6 cells, incubated at 500 μM steroid. Number of experiments: whole cells: $n = 3$; otherwise: $n = 3$–5. n.d.: not determined.

1 mM DTT, 5% sucrose). The microsomal fraction was obtained after centrifugation of the S9 fraction for 60 min at 100 000 × *g*.

2.6. Enzymatic assays and data analysis

Microsomal or S9 fractions were incubated at 37°C for 10–60 min in a reaction mixture containing 0.5 mM coenzyme ($NADP^+$ or NADPH), 1–100 μM steroid substrate, 10–20 μg protein, in 100 mM Tris-HCl, pH 7.4. Inhibitors were used at concentrations ranging from 0.05 to 500 μM. Reactions were stopped by addition of a 3-fold excess of acetonitrile and centrifugation for 5 min. The obtained supernatants were analyzed by RP-HPLC on a C18 stationary phase with an eluent of 30% acetonitrile in 0.1% ammonium acetate, pH 7.0. UV detection of metabolites was achieved at 240 nm. Kinetic constants were calculated using the Enzpack for Windows software (Biosoft, Cambridge, UK). Protein concentrations of samples were determined using the Lowry method with bovine serum albumin as standard.

3. Results

3.1. Cloning and expression of human 11β-HSD-1 in P. pastoris

The human hepatic 11β-HSD-1 was cloned into the expression plasmid pPIC3.5, transformed into *P. pastoris* strains GS115 and KM71, and clones expressing recombinant protein were selected.

3.2. Specific activities in liver and yeast microsomes

Yeast microsomal activities were about 8–10-fold higher than those of human liver microsomes, which is the tissue with the highest in vivo specific activity (Table 1). This indicates successful overexpression of the human gene in *P. pastoris*. However, whereas an enrichment in specific activity of the oxidative reaction is achieved during microsome preparation from the S9 fraction (5.6–16.6 nmol/min × mg, with an average recovery of 92–97%), a loss in the specific activity of the reductive component occurs, indicating a unidirectional activity loss (0.75 vs. 0.55 nmol/min × mg in S9 and microsome fractions, respectively).

3.3. Glucocorticoid metabolism in whole cells

Whole cells were incubated under aerobic conditions with different amounts of cortisone or cortisol. After extraction, steroid analysis of the medium was performed. Whereas no oxidation of cortisol to cortisone was observed, the reductive reaction was carried out with 0.38 nmol cortisol formed per h per 10^6 cells (Table 1), indicating steroid transport and metabolism in intact recombinant yeast cells.

3.4. Substrate and inhibitor screening

A substrate screening was performed and kinetic constants were determined (Table 2). Among the steroids tested, the naturally occurring GCs (cortisol, cortisone, corticosterone and 11-dehydrocorticosterone) turned out to be good substrates as indicated by their intrinsic clearance (V_{max}/k_m) values. Among the synthetic glucocorticoids, only the ring A unsaturated GCs, prednisone and prednisolone, were metabolized by 11β-HSD. Further substitutions, like 9α-fluorination or ring D modifications as in dexamethasone, deflazacort and budesonide, efficiently prevented 11β-hydroxy dehydrogenation. Bulky substituents at ring D also did not allow 11-oxo reduction as demonstrated by the lack of product formation in the case of 11-oxo budesonide.

The inhibitory effect of synthetic steroids and other compounds was evaluated on the cortisol oxidative and cortisone

Table 2
Kinetic constants of recombinant human 11β-HSD-1 expressed in *P. pastoris*

Substrate	Reaction	K_m	V_{max}	Intrinsic clearance
Steroids				
Cortisol	d	9.2 ± 0.8	5.6 ± 0.2	0.60
Cortisone	r	1.9 ± 0.1	0.75 ± 0.1	0.39
Corticosterone	d	5.9 ± 0.3	1.06 ± 0.1	0.17
11-DHC	r	1.7 ± 0.2	0.63 ± 0.1	0.37
Prednisolone	d	25.7 ± 1.5	5.11 ± 0.2	0.19
Prednisone	r	20.6 ± 1.4	1.38 ± 0.2	0.07

S9 preparations containing recombinant human 11β-HSD-1 were incubated with varying amounts of substrates in the presence of 1 mM coenzyme; metabolite detection and quantitation was achieved by RP-HPLC. No background activities were observed in the parental mock-transformed strain. Intrinsic clearance is defined as V_{max}/K_m as apparent first order rate constant. Other synthetic glucocorticoids tested for 11β-hydroxysteroid dehydrogenase or 11-oxo reductase activities include dexamethasone, budesonide, 11-oxo budesonide and deflazacort, however no activity was detectable.
d: 11β-OH dehydrogenation; r: 11-oxo reduction. Number of experiments: $n = 3$–5.

Table 3
Inhibition constants (K_i; in μM) for 11β-hydroxysteroid dehydrogenase (11β-HSD, substrate: cortisol) and 11-oxo reductase (substrate: cortisone) activities of recombinant human 11β-HSD-1

Inhibitor	11β-HSD	11-Oxo reductase
Dexamethasone	12.3 ± 2.5	8.4 ± 0.5
Budesonide	2.8 ± 0.3	58.3 ± 7.9
Deflazacort	2.4 ± 0.3	73.6 ± 17.1
Stanozolol	2.1 ± 0.2	4.05 ± 0.8
Glycerrhitinic acid	0.35 ± 0.09	1.87 ± 0.3
Carbenoxolone	0.04 ± 0.01	0.017 ± 0.001
Naringenin	350 ± 30.5	327 ± 25.0
Furosemide	0.33 ± 0.05	0.55 ± 0.1

Values are given as mean ± S.D. Number of experiments: $n = 3–4$.

reductive activities of the recombinant enzyme (Table 3). Strong inhibition ($K_i < 2$ μmol/l) was observed with glycerrhitinic acid (GA), its hemisuccinate ester carbenoxolone (CBX), and the diuretic compound furosemide. Intermediate inhibition (2 μmol/l $< K_i <$ 100 μmol/l) was observed with synthetic steroids (dexamethasone, budesonide, deflazacort and stanozolol), and weak inhibition with the flavonoid naringenin, which displayed comparatively high K_i values (350 and 327 μmol/l for oxidation and reduction, respectively).

4. Discussion

Several investigations have established the importance of the 11β-hydroxysteroid dehydrogenase system in the regulation of corticosteroid hormone action, achieved by controlling the access of 'active' cortisol to mineralocorticoid and glucocorticoid receptors [4,10,13–15]. Clear evidence has been obtained for a crucial function of the type 2 enzyme (11β-HSD-2) in mineralocorticoid receptor protection by oxidation of glucocorticoids [10,16]. Recent experimental data suggest a basic role for the type 1 enzyme in regenerating 'active' cortisol by reduction from cortisone, essential in glucocorticoid functions such as hepatic carbohydrate metabolism and adipose tissue fatty acid metabolism [13–15]. Analyses of homozygous mice deficient in a functional type 1 11β-HSD gene revealed resistance of these animals against hyperglycemia provoked by stress or obesity [15]. Furthermore, 11β-HSD-1 expression in adipose tissues indicates a central position of this enzyme in the pathogenesis of obesity, hypertension and diabetes [13,14]. Thus, blockade by specific inhibitors might be advantageous in the pharmacological intervention towards these diseases, a concept currently pursued.

Transient expression of 11β-HSD-1 in mammalian cell culture systems has been reported [9,17–20] with low specific activities. However, no successful bacterial, yeast or insect cell overexpression of 11β-HSD-1 has been documented. To allow studies on structure-function relationships of SDR hydroxysteroid dehydrogenases [6,21] we performed expression of the human 11β-HSD-1 in the yeast *P. pastoris* and determined substrate specificities and inhibitor constants using synthetic and naturally occurring compounds.

Data obtained from our enzymological analyses verified that the recombinant enzyme expresses kinetic constants consistent with other data [17]. Recombinant human 11β-HSD-1 behaves in the yeast cellular environment as it does in mammalian systems (Table 1), i.e. when whole cells are incubated only reductive activity is found [9,20]. Upon cell disruption, the oxidative component is observed, exceeding the reductive activity, which appears to be more labile than the oxidative activity. Such a selective difference in reaction direction of a hydroxysteroid dehydrogenase is also consistent with the results of mutagenesis screenings, showing the critical dependence of certain residues for the reaction direction [21].

Metabolism of synthetic glucocorticoids, clinically used for immune suppression, is an important determinant of their action. Depending on their chemical structure, they display pharmacological profiles different from those of the naturally occurring glucocorticoids. This can be explained in part by their different metabolic properties, as for example in the case of 9α-fluorinated GCs [22,23]. We therefore studied the metabolism and inhibitor characteristics of commonly used synthetic systemic and topical GCs with the recombinant type 1 enzyme, allowing us to draw conclusions concerning the active site architecture. Steroid ring A modifications, such as a double bond between C1 and C2 (prednisone, prednisolone), do not prevent catalysis. However, electronegative groups, such as those from 9α-fluorination in dexamethasone, block the dehydrogenase reaction. The reductase reaction, however, possibly is not affected either in type 1 or in type 2 11β-HSDs [24]. Bulky substituents in the vicinity of ring D, e.g. 17,16-oxazole (deflazacort) or 16α,17α- propylmethyleneacetal (budesonide), obstruct catalysis and these compounds are no substrates for 11β-HSD-1. Importantly, the inhibition constants of three compounds, budesonide, deflazacort and GA, were significantly different concerning the reaction direction performed.

These novel findings suggest use of these substances in further analysis of the three-dimensional structure and molecular reaction mechanism of 11β-HSD-1. Naringenin, a flavonoid ingredient of grapefruit juice, inhibits equally reductive and oxidative components of 11β-HSD with K_i values in the μM range, indicating that this compound probably binds with weak affinity to sites other than the steroid substrate, as observed in other studies with various dehydrogenases and flavone compounds [25,26]. Furosemide strongly inhibits both reactions, in the same order of magnitude as GA in our assay, indicating species and tissue differences in inhibitor susceptibility [26]. In conclusion, we achieved in this study expression of native recombinant human 11β-HSD-1, demonstrated properties of this protein, and investigated the inhibitor profiles of synthetic compounds with this medically important enzyme.

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