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Research Article

Human and rodent type 1 11β-hydroxysteroid dehydrogenases are 7β-hydroxycholesterol dehydrogenases involved in oxysterol metabolism

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Abstract. Interconversion between cortisone and the glucocorticoid receptor ligand cortisol is carried out by 11β-hydroxysteroid dehydrogenase (11β-HSD) isozymes and constitutes a medically important example of pre-receptor control of steroid hormones. The enzyme 11β-HSD type 1 (11β-HSD1) catalyzes the conversion of cortisone to its active receptor-binding derivative cortisol, whereas 11β-HSD type 2 performs the reverse reaction. Specific inhibitors against the type 1 enzyme lower intracellular levels of glucocorticoid hormone, with an important clinical application in insulin resistance and other metabolic disorders. We report here on the in vitro oxysterol-metabolizing properties of human and rodent 11β-HSD1. The enzyme, either as full-length, membrane-attached, or as a transmembrane domain-deleted, soluble form, mediates exclusively conversion between 7-ketocholesterol and 7β-hydroxycholesterol with similar k_{cat} values as observed with glucocorticoid hormones. Thus, human, rat, and mouse 11β-HSD1 have dual enzyme activities like the recently described 7α-hydroxysteroid dehydrogenase/11β-hydroxysteroid dehydrogenase from hamster liver, but differ fundamentally from the latter in that 7β-OH rather than 7α-OH dehydrogenase constitutes the second activity. These results demonstrate an enzymatic origin of species differences in 7-oxysterol metabolism, establish the origin of endogenous 7β-OH cholesterol in humans, and point to a possible involvement of 11β-HSD1 in atherosclerosis.

Key words. Oxysterol metabolism; glucocorticoid metabolism; 11β-hydroxysteroid dehydrogenase; 7β-hydroxysteroid dehydrogenase; metabolic syndrome.

Oxysterols constitute an important class of oxygenated products of cholesterol with a broad spectrum of biological activities [1]. Oxysterols serve as intermediates in bile acid synthesis pathways and as ligands for nuclear receptors or oxysterol-binding proteins, and thereby regulate essential pathways in bile acid synthesis, fatty acid synthesis, cholesterol transport, and carbohydrate metabolism [1]. Oxygenation reactions occur at different side chain or ring positions of cholesterol or its derivatives, and result from auto-oxidation or specific enzymatic catalysis. Among the different oxysterols, 7-ketocholesterol is one of the most important oxysterols found in human plasma [1–3].

Recently, Song et al. [4, 5] reported the isolation and partial sequence analysis of an $NADP^+$-dependent 7α-OH

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cholesterol dehydrogenase (7α-HSD) from hamster liver. These studies together with the molecular cloning of hamster 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) from our laboratories [M. Hult et al., unpublished data] suggest identity between these two enzymes and possible interactions between oxysterol and glucocorticoid metabolic pathways.

Thus far, two isozymes of 11β-HSD have been characterized: both are members of the conserved superfamily of short-chain dehydrogenases/reductases (SDRs), and both 11β-HSD forms constitute important components of glucocorticoid hormone signaling [6, 7]. Whereas in most instances 11β-HSD1 reduces cortisone (dehydrocorticosterone in rodents) in an NADPH-dependent reaction to the glucocorticoid receptor (GR) ligand cortisol (corticosterone in rodents), the oxidative NAD^+-dependent inactivation of cortisol is carried out by the type 2 isozyme (11β-HSD2) [8]. In this manner, tissue-specific adaptation to intracellular glucocorticoid levels is maintained. The postulated role of these enzymes has been confirmed in 11β-HSD1 transgenic or knock-out animal models [9–12], and by genetic defects in the *11HSD2* gene, leading to juvenile hypertension and electrolyte dysregulation [13, 14]. These in vivo and in vitro studies have demonstrated the importance of amplification of glucocorticoid hormone signaling, carried out by the type 1 isozyme. As a result, modulation of this prereceptor control and ligand metabolism has been identified as a pharmacological target for the treatment of non-insulin-dependent diabetes mellitus and related metabolic disorders [9, 10, 15–17], with concomitant successful development of selective inhibitors [18–20]. In the present report, we investigate the relationships between oxysterol and glucocorticoid metabolism. In particular, the aims were to analyze the oxysterol-metabolizing properties of recombinant 11β-HSD1 from human, mouse, and rat, and to compare their properties to oxysterol activities found in human liver microsomes.

Materials and methods

Materials and chemicals

Human liver microsomes were obtained from a transplant donor, in accordance with the local ethical committee. 7α-OH-cholesterol was purchased from Steraloids, 7β-OH-cholesterol, 7-ketocholesterol, glucocorticoids, and carbenoxolone were obtained from Sigma. Cholesterol oxidase at a specific activity of 24 U/ml from *Nocardia erythopolis* was obtained from BioChemika. BVT.24829 is a derivative of the lead series developed as a selective and tight-binding inhibitor of human 11β-HSD1 (Biovitrum) [18].

Cloning and expression of 11β-HSD1 species variants

Three different 11β-HSD1 species variants (human, rat, mouse) were analyzed. The enzyme preparations used in this study were cell homogenates produced in the yeast *Pichia pastoris* containing recombinant full-length enzymes [21, 22], transmembrane-deleted enzyme versions expressed and purified from *Escherichia coli* [22], and human liver microsomes. Microsomes were collected by differential ultracentrifugation. Constructs coding for 11β-HSD1 were cloned by PCR, expression, preparation of homogenates, and purification were performed essentially as described elsewhere [21–23].

Enzymatic assays and data analysis

The amount of enzyme and incubation time was adjusted to be in the linear range of product formation. All reactions were carried out at 37 °C, in 20 mM Tris/HCl, pH 7.4. In the reactions containing 7-oxo-cholesterol derivatives, 0.1% Triton X-100 was used for solubilization, final reaction solutions were obtained by diluting oxysterol stocks (dissolved in 99% ethanol, v/v, p.a.) with a final ethanol concentration of 2% (v/v). For HPLC assays, the reaction volumes were 50 µl; in the fluorimetric assays, volumes were 320 µl.

Enzymatic tests for dehydrogenase activities (7α-hydroxycholesterol (7α-OH-chol), 7β-hydroxycholesterol (7β-OH-chol), cortisol, corticosterone)) using protein homogenates from human liver and *P. pastoris* were performed at 20 µM substrate concentration, 20 µM $NADP^+$. Reactions were terminated with 1 reaction volume of acetonitrile, and insoluble protein was removed by centrifugation. Enzymatic tests for reductase activities (7-ketocholesterol, cortisone, dehydrocorticosterone) were measured with 20 µM substrate and an NADPH-regenerating system [0.5% dilution of a mixture containing 12 mM NADP+, 29 g/l glucose-6-phosphate, 5 mM $MgCl_2$, 0.2 M Tris/HCl, pH 7.4, 8.5 U/ml glucose-6-phosphate dehydrogenase (Roche)], with or without inhibitor. The 7-ketocholesterol reactions were terminated by heating at 60 °C for 3 min. The enzymatically formed 7-OH-product was converted into 7-OH-4-cholestene-3-one by addition of 120 mU cholesterol oxidase, subsequent incubation at 37 °C for 20 min, followed by termination with acetonitrile. Forty microliters of each terminated enzymatic reaction was analyzed for conversion by RP-HPLC with a C18 column. Individual experiments were carried out with single data points. The mobile phase was 30% acetonitrile (v/v), 17 mM ammonium acetate, pH 7.0, (glucocorticoid separation) or 85% acetonitrile/water (v/v, 7-cholesterol derivative separation). The glucocorticoid absorption was measured at 240 nm, and 7-keto-cholesterol and 7-OH-4-cholestene-3-one absorption was measured at 241 nm. The amount of product formed was determined by comparison to external standard curves of authentic 7-oxo-cholesterol derivatives.

Determinations of 7α-OH-chol, 7β-OH-chol and 11β-HSD activities (cortisol, corticosterone) using purified 11β-HSD1 were performed at 25 µM substrate concentration and 50 µM $NADP^+$. NADPH formation was measured continuously for 20–30 min in a Fluoroskan Ascent microplate fluorimeter (Labsystems) (excitation 340 nm, emission 460 nm). 7β-OH-chol dehydrogenase kinetic reactions were performed at varied substrate concentrations (0.8–100 µM) and 50 µM $NADP^+$. The relationship between the increase of fluorescence and the rate of NADPH formation was established by generating a calibration curve using freshly prepared solutions of NADPH (0–25 µM). Kinetic constants were calculated using the PRISM (GraphPad) or GRAFIT (Erithacus Software) software packages by non-linear regression analysis of data fitted to the Michaelis-Menten kinetics function.

Active-site titration

Active-site titration of 11β-HSD1-containing *P. pastoris* homogenates or purified soluble enzyme was performed as described using carbenoxolone (CBX) or the specific tight-binding inhibitor BVT.24829 [22]. Data were fitted by non-linear regression to the Morrison equation [24], thus obtaining apparent K_i and enzyme concentration values.

Results

Metabolism of 7-oxysterols in human liver microsomes

Human liver microsomes were incubated in the presence of NADP(H) and either 7α-OH-chol, 7β-OH-chol or 7-ketocholesterol, and metabolite formation was assessed by RP-HPLC (fig. 1). These experiments revealed that 7-ketocholesterol is reduced exclusively in human liver microsomes to 7β-OH-chol using NADPH, without detectable formation of 7α-OH-chol. Conversely, only 7β-OH-chol but not 7α-OH-chol is oxidized in vitro to the 7-oxo derivative by human liver microsomes. Quantitative analysis of a sample from a single human transplant donor (table 1) demonstrates detectable levels of both 11β-HSD and 7β-OH-chol dehydrogenase activities. For both enzyme activities, the rate of ketoreductase activity was four- to eightfold higher than the opposing dehydrogenase activity. To establish relationships with hepatic 11β-HSD1, inhibition experiments were performed. In these experiments, the 11β-HSD inhibitor CBX yielded close to complete inhibition of 7-oxysterol metabolite formation in microsomes, suggesting a possible identity of human hepatic 11β-HSD1 with 7β-OH-chol dehydrogenase (table 1, fig. 1). Using a recently developed specific inhibitor for type 1 11β-HSD (BVT.24829) [18–20], complete inhibition of glucocorticoid conversion was achieved; however, a residual 7-ketocholesterol activity was apparent after inhibition. These inhibition data suggest an additional, different 7-ketocholesterol reductase enzyme other than 11β-HSD1 with low activity present in microsomes, or a minor amount of non-enzymatic autoconversion.

Table 1. 7-Oxysterol and glucocorticoid metabolism in human liver microsomes.

Substrate	Activity	+ CBX residual activity (%)	+ BVT.24829 residual activity (%)
Cortisol	0.56 ± 0.02	0	0
7α-OH-chol	na	–	–
7β-OH-chol	0.08 ± 0.01	0	0
7-Ketocholesterol	0.68 ± 0.03	2	6
Cortisone	2.3 ± 0.1	0	0

Activity: pmol × min^{-1} × mg^{-1}, residual activity in % cf. uninhibited experiments, which were set to 100%. na, no activity detectable. Reaction rates were measured at substrate (cortisol/7α-OH-chol/7β-OH-chol) concentration of 20 µM for dehydrogenase activity, the reductase reaction was measured at 20 µM 7-ketocholesterol/cortisone; and inhibitor concentrations were 2.5 µM CBX and BVT.24829. Activity is presented as mean ± SD, n = 3 independent experiments for each value, except for cortisone and cortisone/CBX, where n = 2. Incubation times 60 min for 7keto/7α-OH/7β-OH-chol, 30 min for cortisol, and 15 min for cortisone.

Recombinant full-length 11β-HSD1 from human, mouse, and rat are exclusive 7β-OH-chol dehydrogenases

Recombinant 11β-HSD1 species variants were expressed as full-length forms in the methylotrophic yeast *P. pastoris*. These variants of 11β-HSD1 from human, rat, and mouse showed qualitatively the same pattern of conversion of 7-oxysterols as observed with the human liver microsome material (table 2). In particular, no product formation was observed with 7α-OH-chol as substrate. However, reactions with 7β-OH-chol and $NADP^+$ led to 7-ketocholesterol formation. In all cases, CBX inhibition resulted in a complete decrease in activity, clearly indicating mediation of 7-oxysterol conversion by the recombinant full-length 11β-HSD1 variants.

Enzymatic properties of purified 11β-HSD1 species variants

11β-HSD1 versions from human, mouse, and rat were expressed in *E. coli* as soluble variants lacking the transmembrane domain, allowing purification by metal affinity chromatography through N-terminal His_6 tags. In previous studies, we reported on the kinetic characteristics using glucocorticoids as substrates of the human and guinea pig forms, and on the purification and functional expression of these different variants expressed in *E. coli* and *P. pastoris* [21–23]. In those studies, we established equivalence between the transmembrane-deleted and the full-length versions regarding their kinetic behavior to-

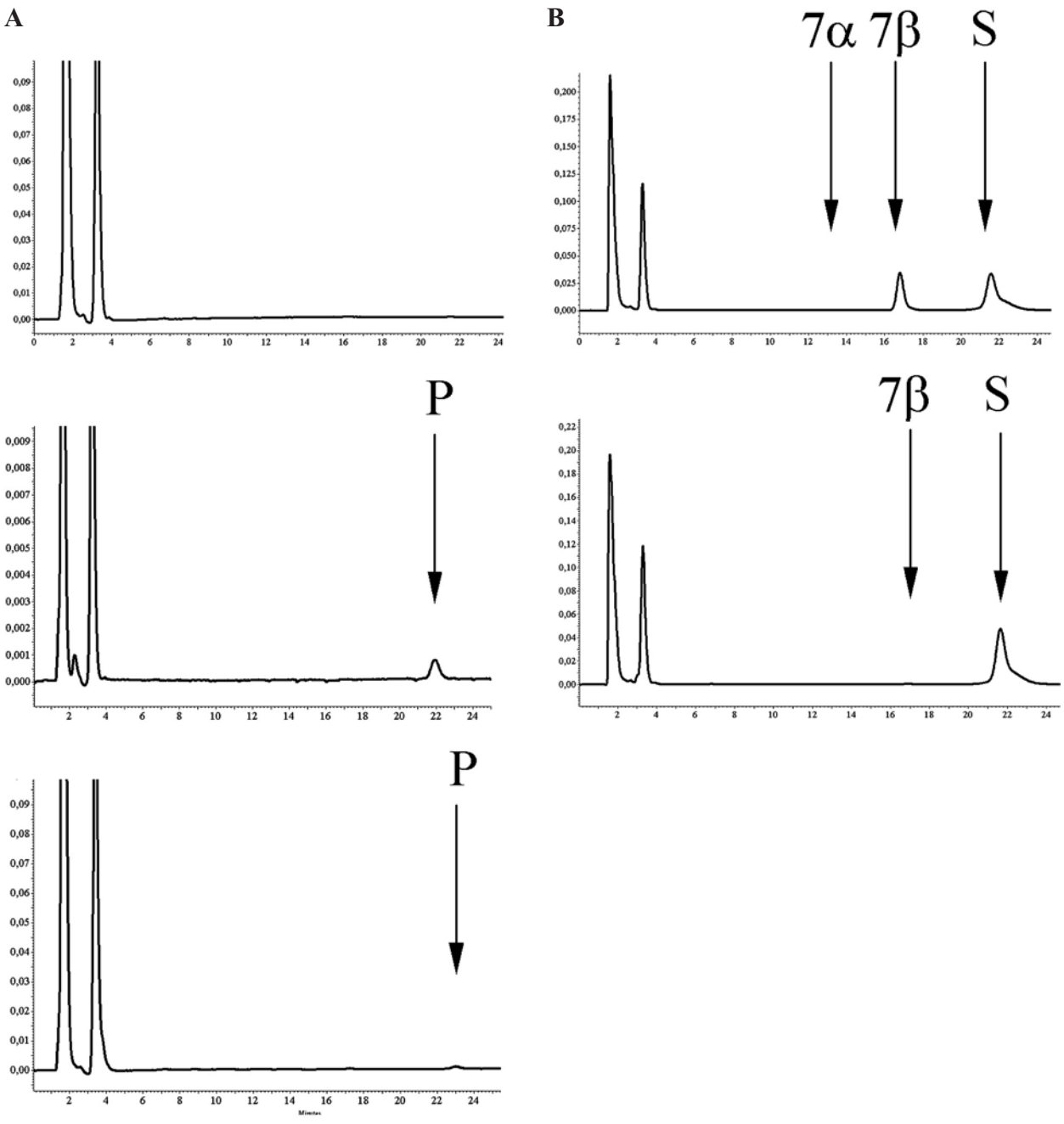

Figure 1. NADP(H)-dependent formation of 7-oxysterol metabolites in human liver microsomes. (*A*) Dehydrogenase reactions, from top to bottom: RP-HPLC chromatograms and product identifications from reactions with $NADP^+$, 7α-OH-chol (top); $NADP^+$, 7β-OH-chol (middle); $NADP^+$, 7β-OH-chol, CBX (bottom). Only 7β-OH-chol, $NADP^+$ leads to 7-ketocholesterol (P) formation. (*B*) Reduction reactions: NADPH, 7-ketocholesterol; production of 7β-OH-chol (7β) but not 7α-OH-chol (7α) from 7-ketocholesterol (S) (top); NADPH, 7-ketocholesterol, CBX; inhibition of product formation by CBX (bottom).

Table 2. Formation of 7-ketocholesterol from 7α-OH- and 7β-OH-chol as substrate using full-length 11β-HSD1 species variants expressed in *P. pastoris*.

Species	7α-OH-chol	7α-OH-chol/CBX residual activity (%)	7β-OH-chol	7β-OH-chol/CBX residual activity (%)
Human	na	–	0.41 ± 0.03	0
Mouse	na	–	0.88 ± 0.35	0
Rat	na	–	0.07 ± 0.01	0
Control	na	–	na	–

Values of $k_{cat,app}$ (1/min) were determined at saturating substrate concentrations, and enzyme concentrations were determined by active-site titration. na, no activity detectable. Activity is presented as mean ± SD; n = 2 independent experiments. Incubation time 60 min. Control, mock-transformed *P. pastoris*.

Table 3. Kinetic parameters of purified soluble human, rat and mouse 11β-HSD1 species variants.

	7β-OH dehydrogenase			7-oxo reductase		
	K_m	k_{cat}	k_{cat}/K_m	K_m	k_{cat}	k_{cat}/K_m
Human	17 ± 2	7.4 ± 1.1	0.46	51 ± 12	1.2 ± 0.3	0.03
Mouse	1.5 ± 0.5	4.8 ± 0.8	3.3	7.7 ± 1.2	7.0 ± 0.8	0.9
Rat	11 ± 2.4	22 ± 1.4	2.0	85 ± 18	5.1 ± 1.5	0.06

K_m in µM, k_{cat} in min^{-1}, k_{cat}/K_m in $\mu M^{-1} \times min^{-1}$. Activities are presented as mean SD; n = 4 independent experiments for human, n = 2 for mouse, n = 2 for rat reductase, and n = 3 for rat dehydrogenase activity. Incubation times: 30 min.

ward their glucocorticoid substrates. In the present study, we performed active-site titration experiments using CBX to determine the exact amount of catalytically competent enzyme in the enzyme preparations, in a manner analogous to that described for the human enzyme using a tight-binding arylsulfonamidothiazole derivative [22]. With the three species investigated, CBX showed tight-binding inhibition using glucocorticoids as substrates. This allowed us to derive k_{cat} values, instrumental for comparison of catalytic efficiencies. All three soluble 11β-HSD species variants investigated showed qualitatively the same oxysterol conversion pattern as their full-length variants (described above), but differed in their kinetic constants (table 3). Resulting k_{cat}/K_m calculations established that the mouse form most efficiently catalyzes 7β-OH/7-keto conversions, followed by rat, and human. Kinetic analysis revealed Michaelis-Menten-type behavior (fig. 2).

Inhibition experiments were performed to evaluate the possible interference of oxysterol inhibition on glucocor-

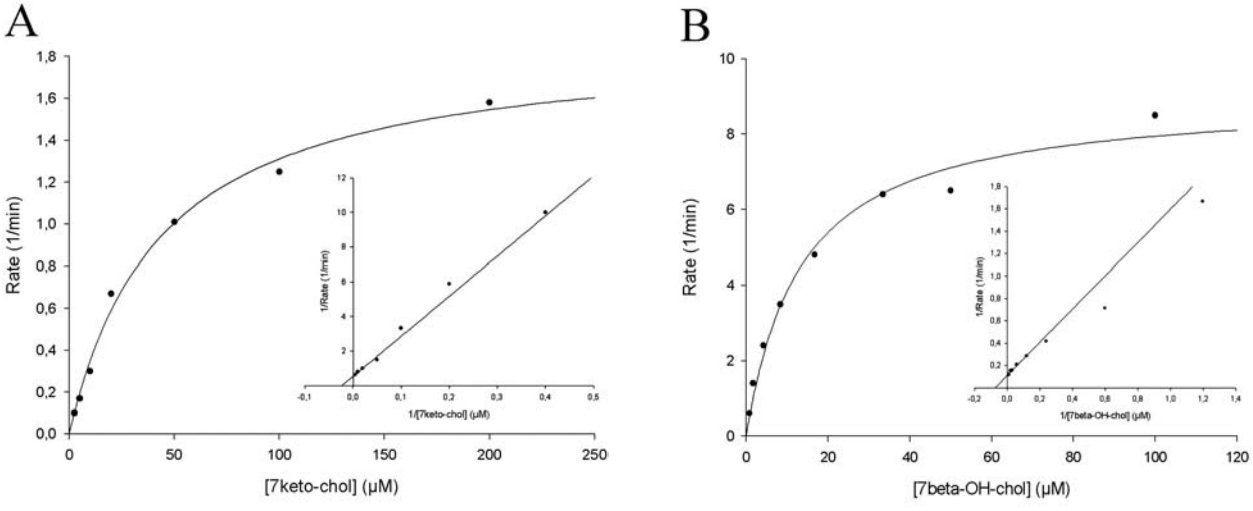

Figure 2. Representative kinetic analysis of purified human 11β-HSD1, demonstrating Michaelis-Menten kinetics with 7-oxysterols. Data were fitted by non-linear regression to the Michaelis-Menten equation. Inserts show plots of data transformed by Lineweaver-Burke linear regression. (*A*) 7β-OH-chol dehydrogenase reaction. (*B*) 7-Ketocholesterol reductase reaction.

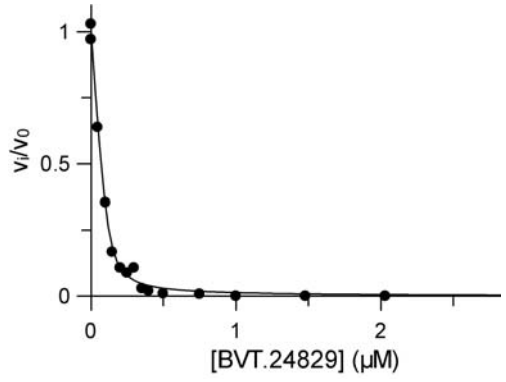

Figure 3. Inhibition of 7-oxysterol conversion by the selective 11β-HSD1 inhibitor BVT.24829. Inhibition of 7β-OH-chol dehydrogenase activity was carried out at varied inhibitor concentrations. Duplicate measurements were made at 0 M BVT.24829 to give an approximate measure of the variation expected in the other plotted points. Non-linear regression analysis of the data obtained [plot of inhibitor concentration against fractional velocity (v_i/v_0)] reveals an apparent K_i of 12 nM. Independent duplicate measurements, performed to avoid occasional error, agreed to within 10%. NADPH formation was measured continuously at 6-s intervals (20 min total time) by the fluorimetric assay as described in Materials and methods.

ticoid conversion by purified human 11β-HSD1. We tested possible inhibition by 7α-OH-chol, 7β-OH-chol and 7-ketocholesterol. In these experiments, we did not observe a strong inhibition by the 7-oxysterols tested, neither of the physiologically relevant 11-oxo-reductase nor of the 11β-dehydrogenase reaction, with K_i values ranging from 30 to 240 μM (data not shown), in line with the observed kinetic data. However, efficient inhibition of the 7β-OH-chol dehydrogenase activity using the arylsulfonamidothiazole BVT.24829 was observed (fig. 3). Experiments were conducted at 50 μM substrate concentration, resulting in an apparent K_i of 12 ± 2 nM.

Discussion

Recent studies [4, 5] indicated a possible relationship between oxysterol and glucocorticoid hormone metabolism through the discovery of a hamster liver 7α-OH dehydrogenase, displaying a high similarity to hepatic 11β-HSD1 cloned from other species [4, 5]. We have now established that human, rat, and mouse 11β-HSD1 species variants function as 7β-OH-chol dehydrogenase, in contrast to the hamster isozyme which appears to mediate mainly 7α-OH activity [4, 5]. Our data using recombinant 11β-HSD1 are in perfect agreement with the conversion pattern found in human liver microsomes, demonstrating solely 7β-OH activity. Using two different inhibitors, the less specific steroid and prostaglandin dehydrogenase inhibitor CBX, and the 11β-HSD1-specific inhibitor BVT.24829 [18–20], we discovered that 11β-HSD1 is the main enzyme mediating oxidoreductase reactions at position 7 of 7-oxysterols in human liver microsomes. We also found in CBX and BVT.24829 inhibition experiments some minor (about 2–6%) ketocholesterol reductase activity, which could not be attributed to 11β-HSD1-mediated conversion.

To date, no analysis of oxysterol metabolism has been carried out in 11β-HSD1 animal models [9–12]. Furthermore, data obtained from knock-out or transgenic animals are difficult to interpret and to extrapolate with respect to development of atherosclerosis in humans, since cholesterol and lipoprotein metabolism show a high degree of species variability [1]. However, bearing in mind the established 7-keto reductase activity of 11β-HSD1 (this report) and its widespread expression pattern [8], one can now explain earlier results demonstrating in vivo and in vitro formation of 7β-OH-chol from 7-ketocholesterol [25, 26]. Given the well-documented preference of 11β-HSD1 to mediate the reductive reaction in vivo and in intact cells [27, 28], and given the in vivo formation of 7β-OH-chol in studies using radiolabeled 7-ketocholesterol [26], one may anticipate that formation of 7β-OH-chol from 7-ketocholesterol is catalyzed mainly by 11β-HSD1 in the liver, a major site of oxysterol in vivo metabolism. The issue that still remains to be considered is the high K_m value observed with the purified enzyme material. Although large species variations were noted in our study, the values obtained are at least one order of magnitude above the plasma concentrations reported for 7β-OH-chol and 7-ketocholesterol [1]. However, our data were obtained in vitro under artificial conditions and by using enzyme devoid of membrane attachment. Furthermore, the local effective concentrations of oxysterols in the endoplasmic reticulum (ER) membrane, in the vicinity of 11β-HSD1, are unknown. Given the hydrophobicity of cholesterol derivatives and their enrichment in lipid membranes, we assume a considerably higher effective concentration than values based on plasma levels or K_m data obtained in in vitro systems. Whatever the concentrations of the oxysterols in the ER membrane are, the in vivo experiments indicate clearly that effective conversion of 7-ketocholesterol to the 7β-OH-chol derivative occurs, e.g., in rat liver [26].

The possible in vivo role of 7-oxysterols is largely unknown, but documented biological effects of 7-oxysterols range from induction of apoptosis in macrophage-like cell lines [29], accumulation in atherosclerotic lesions, involvement in lipid peroxidation [30], and induction of foam cell formation [31]. Despite the apparently high concentrations of oxysterols used in some of these experiments, the data suggest a possible role of 7-ketocholesterol in the progression of atherosclerotic disease. The transduction pathways behind these effects may be of multiple origins, involving yet poorly defined membrane effects [29], but could also be mediated by intracellular

nuclear receptors, in analogy with the properties of side-chain-oxygenated cholesterol ligands binding to the liver X receptor [32, 33], showing a critical role in reverse cholesterol transport, inflammation and, hence, atherosclerosis. This notion is further supported by the recent discovery that 7-ketocholesterol binds to the arylhydrocarbon receptor, at concentrations in the physiological range [34].

Assuming a reductive reaction preference of 11β-HSD1, 7-ketocholesterol formed through lipid peroxidation processes as a consequence of oxidative stress will be transformed into the more polar 7β-OH metabolite through 11β-HSD1 in the liver. Although some of the biological activities of 7-oxysterols are also obtained with 7β-OH-chol, plasma levels are low, suggesting rapid further metabolism and excretion. However, the role of 11β-HSD1 in atherosclerotic lesions might be radically different from the scenarios observed in liver. First, 11β-HSD1 expression is observed in smooth muscle cells, additionally during specific stages in macrophage differentiation, and is dependent on specific cytokines such as interleukin IL-4 and IL-13 [35]. This suggests a role of 11β-HSD1 in macrophage pathophysiology in atherosclerotic lesion development, both through modulation of immune-modulatory signals (glucocorticoids) and through metabolism of pro-atherogenic compounds (oxysterols). However, thus far no enzymological analysis of 7-oxysterol conversion at specific points of macrophage development, derived from animal or cell culture models, or atherosclerotic plaques has been reported. Second, the reaction direction of 11β-HSD1 might be differentiation dependent. Data from the group of Stewart et al. [36, 37] suggest important changes in the stage-specific cortisone-cortisol conversion pattern during, e.g., adipocyte differentiation, implying a role of 11β-HSD1 in the pro-differentiating and anti-proliferative properties of glucocorticoids. This mechanism might also be operative in cell types other than adipocytes. In macrophages, a change in reaction direction of oxysterol metabolism (from reductive to oxidative) would imply that 11β-HSD1 prevents inactivation of the pro-atherogenic 7-ketocholesterol and even enhances formation of this oxysterol through 7β-OH dehydrogenation.

Taken together, this study provides novel insights into enzymological properties of an important enzyme, central in glucocorticoid physiology and pharmacology, and points to possible novel functions as an oxysterol-metabolizing enzyme within metabolic disease and atherosclerosis.

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