Hepatic reduction of the secondary bile acid 7-oxolithocholic acid is mediated by 11β-hydroxysteroid dehydrogenase 1

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INTRODUCTION

Bile acids play an important role in the processing and uptake of dietary lipids and fat-soluble vitamins, and in the elimination of cholesterol and toxic lipophilic compounds from the body. Impaired regulation of the composition and concentration of bile acids and bile salts has been associated with hepatobiliary and digestive diseases [1]. Thus it is important to identify the proteins involved in the maintenance of bile acid homeostasis.

Bile acids are synthesized from cholesterol by CYP (cytochrome P450)-mediated oxidative type I biotransformation reactions. In addition to CYP enzymes, oxidoreductases and peroxisomal oxidases are involved in bile acid synthesis [2]. The major bile acids present in human bile are CDCA (chenodeoxycholic acid) (35–50 %), its 12-hydroxylated derivative CA (cholic acid) (30–45 %) and DCA (deoxycholic acid) (10–20 %), the bacterial 7-deoxy metabolite of CA (for structures, see Figure 1) [3,4]. Several type II biotransformation reactions of bile acids occur in the liver. Unconjugated bile acids, either newly synthesized or reaching the liver after bacterial deconjugation via the enterohepatic circulation [5], are subjected either newly synthesized or reaching the liver after bacterial deconjugation via the enterohepatic circulation [5]. Furthermore, hepatic enzymes convert iso- or 3-bile acids into the preferred 3α-hydroxy derivatives and 7-bile acids into the 7α- and 7β-hydroxy forms [6]. Whereas several hydroxylating CYPs and conjugating liver enzymes have been identified and characterized, the enzyme(s) involved in the hepatic oxidation of 7-bile acids remained unknown.

In the human colon, several bacterial strains, including Escherichia coli, Bacteroides fragilis and Bacteroides intestinalis, express 7α-HSD (7α-hydroxysteroid dehydrogenase) enzymes that generate 7-oxoDCA (7-oxodeoxycholic acid) from CA, and 7-oxoLCA (7-oxolithocholic acid) from CDCA and UDCA (ursodeoxycholic acid) [7,8]. The gut microbiota also contains hydroxysteroid dehydrogenases that catalyse the epimerization of 7α- to 7β-hydroxy bile acids with the generation of a stable oxo bile acid intermediate [9–11], thus contributing to the formation of the UDCA found in bile and faeces. The secondary bile acids DCA and LCA, as well as the 7-oxo bile acids 7-oxoDCA and 7-oxoLCA, are formed primarily by gut micro-organisms, is reduced in human liver to CDCA (chenodeoxycholic acid) and, to a lesser extent, UDCA (ursodeoxycholic acid). The enzyme(s) responsible remains unknown. Using human liver microsomes, we observed enhanced 7-oxoLCA reduction in the presence of detergent. The reaction was dependent on NADPH and stimulated by glucose 6-phosphate, suggesting localization of the enzyme in the ER (endoplasmic reticulum) and dependence on NADPH-producing H6PDH (hexose-6-phosphate dehydrogenase). Using recombinant human 11β-HSD1 (11β-hydroxysteroid dehydrogenase 1), we demonstrate efficient conversion of 7-oxoLCA into CDCA and, to a lesser extent, UDCA. Unlike the reversible metabolism of glucocorticoids, 11β-HSD1 mediated solely 7-oxo reduction of 7-oxoLCA and its taurine and glycine conjugates. Furthermore, we investigated the interference of bile acids with 11β-HSD1-dependent interconversion of glucocorticoids. 7-OxoLCA and its conjugates preferentially inhibited cortisone reduction, and CDCA and its conjugates inhibited cortisol oxidation. Three-dimensional modelling provided an explanation for the binding mode and selectivity of the bile acids studied. The results reveal that 11β-HSD1 is responsible for 7-oxoLCA reduction in humans, providing a further link between hepatic glucocorticoid activation and bile acid metabolism. These findings also suggest the need for animal and clinical studies to explore whether inhibition of 11β-HSD1 to reduce cortisol levels would also lead to an accumulation of 7-oxoLCA, thereby potentially affecting bile acid-mediated functions.

Key words: bile acid, glucocorticoid, 11β-hydroxysteroid dehydrogenase, liver, metabolism, 7-oxolithocholic acid.
7-oxoLCA, are eliminated by the faeces. However, a substantial fraction of these secondary bile acids is not excreted, but reabsorbed in the distal intestine and transported back to the liver. Early studies on the metabolism of radio-labelled 7-oxoLCA in rats with bile fistulas indicated the preferential formation of UDCA and lower amounts of CDCA and its metabolites [12]. Later, Fromm et al. [13] reported the preferential conversion of radio-labelled 7-oxoLCA into CDCA, with approximately 10% UDCA after a single hepatic passage following i.v. (intravenous) administration in humans. Similarly, 7-oxoLC-Gly (7-oxolithocholylglycine) and 7-oxoLC-Tau (7-oxolithocholyltaurine) were converted into the 7α-hydroxy epimer. After small intestinal infusion, 7-oxoLCA was metabolized primarily to CDCA as observed after i.v. injection. CDCA and UDCA were not metabolized by the liver, suggesting that UDCA is mainly produced by bacterial enzymes as a result of the epimerization of CDCA via the 7-oxoLCA intermediate [9,14,15]. Using human liver preparations Amuro et al. [16] provided evidence that 7-oxoLCA is primarily reduced to CDCA and lower amounts of UDCA by NADPH-dependent microsomal enzyme(s).

In order to identify this missing link, i.e. the source of hepatic reduction of bacterially derived 7-oxoLCA and formation of UDCA, we aimed in the present study to identify the hepatic 7-oxo bile acid reductase. Initially, human liver microsomes and lower amounts of UDCA by NADPH-dependent microsomal enzyme(s).

![Figure 1: Structures of bile acids](image)

**Figure 1** Structures of bile acids

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**Experimental**

**Microsomal preparations and activity assays using human liver microsomes**

Human liver microsomes (InVitro CYP H-class™ microsomes from a male donor, Celsis International) were thawed on ice and used immediately for activity assays. Microsomes, 0.2 mg per reaction, were incubated at 37°C for 0–40 min in a total volume of 500 μl containing TS2 buffer (100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM MgCl₂, 250 mM sucrose and 20 mM Tris/HCl, pH 7.4), a final concentration of 500 μM NADPH, 1 μM substrate and vehicle or 5 μM 11β-HSD1 inhibitor as indicated. To assess the latency of 7-oxoLCA reduction and dependence on cofactor regeneration, microsomes were incubated in reaction mixture supplemented with the detergent Nonidet P40 (final concentration of 0.5%), G6P (glucose 6-phosphate) or G6S (glucose 6-sulfate) (final concentration of 1 mM), or the glucose-6-phosphate translocase inhibitor S3483 (obtained from Sanofi-Aventis; final concentration of 20 μM). Reactions were started by adding microsomes into freshly prepared reaction mixture and stopped by rapid freezing in solid CO₂.

Rat liver microsomes and microsomes of HEK (human embryonic kidney)-293 cells transfected with human 11β-HSD1 or mock-transfected were prepared as described previously [23]. For immunoblotting, 50 μg of total microsomal proteins was separated by 12% PAGE, followed by transfer of proteins on to PVDF membranes. 11β-HSD1 was detected using primary anti-(human 11β-HSD1) antibody (Cayman Chemical).

**Reduction of 7-oxoLCA by recombinant human 11β-HSD1**

To assess 7-oxoLCA reductase activity in intact HEK-293 cells stably expressing recombinant human 11β-HSD1 or co-expressing 11β-HSD1 and H6PDH (AT6 and HHH7 clones respectively) [24], 20000 cells per well were distributed in 24-well plates and allowed to adhere for 16 h. Cells were then incubated with doubly charcoal-treated DMEM (Dulbecco’s modified Eagle’s medium), and the 7-oxideduction of 7-oxoLCA was measured at a final concentration of 1 μM after incubation for up to 24 h at 37°C in a total volume of 1 ml. To determine the apparent Kᵣ of 11β-HSD1, frozen cell lysates were thawed, sonicated and immediately incubated for 10 min at 37°C in a total volume of 500 μl containing 500 μM NADPH and 7-oxoLCA at concentrations between 62.5 nM and 4 μM. Reactions were terminated by freezing in solid CO₂.

**Impact of bile acids on the interconversion of glucocorticoids**

The conversion of cortisone into cortisol using cell lysates was measured as described previously [25] (see the Supplementary Online Data at http://www.BiochemJ.org/bj/436/bj4360621add.htm). To assess the effect of 7-oxoLCA on the ratio of active to inactive glucocorticoids in intact cells at steady state, cells (30 000 cells per well of a poly-L-lysine-coated 96-well plate, Becton-Dickinson) expressing 11β-HSD1 or co-expressing 11β-HSD1 and H6PDH were incubated for 24 h in a total volume of 40 μl of steroid-free DMEM in the presence of either 200 nM cortisone or cortisol and various concentrations of 7-oxoLCA.

**Analysis of non-labelled steroids and bile acids by LC (liquid chromatography)–MS**

Frozen samples from reactions using intact cells, cell lysates or microsomes were thawed, and a fixed amount of deuterated
CDCA (0.5 nmol) or corticosterone (0.2 nmol) was added as an internal standard, followed by mixing and centrifugation at 3000 g for 5 min. Supernatants were loaded on to Oasis HBL SPE cartridges (pre-conditioned with 1 ml of methanol and 1 ml of water), followed by washing with 2 ml of water and elution with 2 ml of methanol. The solvent was evaporated and the residue was reconstituted in 100 μl of methanol.

7-OxolCA and its metabolites were separated on an Atlantis T3 column (Waters) using an Agilent Technologies model 1200 liquid chromatograph (see the Supplementary Online Data). The liquid chromatograph was interfaced to an Agilent 6410 triple-quad mass spectrometer, operated in atmospheric pressure electrospray positive-ionization mode. Data acquisition was performed using MassHunter workstation software (version B.01.04).

Metabolites were identified by comparing their retention times and mass to charge ratios (m/z) with those of authentic standards. UDCA, 7-oxoLCA and CDCA were detected in the selected positive-ionization MS Scan2 mode (mass range, m/z 300–500). They were typically eluted at 5.2, 6.2 and 7.3 min, and were monitored at m/z 357.3, 373.2 and 357.3 (dehydrated bile acids) respectively. Quantitative determination of bile acids was performed by positive-ionization and MRM (Multiple Reaction Monitoring). Deuterated CDCA was used as internal standard (m/z 361.2).

Quantitative analysis of glucocorticoids was performed similarly by MRM. Cortisone (precursor and product ion at m/z 361 and 163) and cortisol (precursor and product ion at m/z 363 and 121) were eluted at 9.8 and 9.5 min respectively. Corticosterone (m/z 347.2) was used as internal standard and was eluted at 12.1 min.

Metabolites were quantified from calibration curves of the peak area ratio of the authentic standard and internal standard incubated in lysates of untransfected HEK-293 cells at a total protein concentration identical with that of the experimental setting and plotted against the concentration of authentic standards (normalization using internal standard).

Figure 2 Reduction of 7-oxoLCA by human liver microsomes

(A) Human liver microsomes were incubated for 40 min with 7-oxoLCA (1 μM), NADPH (500 μM) and either vehicle or hexose 6-phosphate (1 mM G6P or G6S) and 11β-HSD1 inhibitor (5 μM glycyrrhetinic acid (GA), Merck-544 (T0504) or BNW16) as indicated. Samples were analysed for the amount of unconverted substrate 7-oxoLCA (white bars) and the products CDCA (black bars) and UDCA (hatched bars). Results (n = 3) are means ± S.D. (B) Expression of 11β-HSD1 in liver microsomes and in microsomes of transfected HEK-293 cells. A total amount of 50 μg of microsomal proteins was separated by gel electrophoresis, and proteins were blotted on to PVDF membranes and probed with an anti-(human 11β-HSD1) antibody. Asterisks indicate non-specific bands, and arrows indicate 11β-HSD1. Molecular masses are indicated in kDa.

Calculation of enzyme kinetic parameters

Enzyme kinetics was analysed by non-linear regression using four-parameter logistic curve fitting. For statistical comparisons, the ratio t-test in GraphPad Prism 5 software was used. Results (means ± S.D.) were obtained from at least three independent experiments.

Molecular modelling of 11β-HSD1 with bile acids

Mouse 11β-HSD1 (PDB code 1Y5R) [26] was extracted from the PDB for use as a template to investigate the interactions of 7-oxo, 7α-hydroxy and 7β-hydroxy bile acids with 11β-HSD1. We used PDB code 1Y5R because it contains both corticosterone and NADP+, unlike other three-dimensional structures of 11β-HSD1 in the PDB. Human and mouse 11β-HSD1 have 79% sequence identity, which allows mouse 11β-HSD1 to be a good model for the interaction of bile acids with human 11β-HSD1.

To obtain 11β-HSD1 complexed with 7-oxoLCA, we super-imposed the crystal structure of E. coli 7α-HSD [27] complexed with 7-oxoLC-Gly (PDB code 1FMG) with PDB code 1Y5R and extracted 7-oxoLC-Gly from PDB code 1FMG. Then we inserted 7-oxoLCA into 11β-HSD1. For conversion of the 7-oxo into either a 7α-hydroxy or a 7β-hydroxy, we used the Biopolymer option in Insight II. The glycine-conjugated bile acids also were constructed with Biopolymer. The final three-dimensional model of 11β-HSD1 with each bile acid was refined using Discover 3, which was run for 10000 iterations with a distant dependent dielectric constant of 2.

RESULTS

Reduction of 7-oxoLCA by human liver microsomes

Previous studies provided evidence for the existence of one or more hepatic enzymes catalysing the 7-oxo reduction of 7-oxoLCA to CDCA and/or UDCA [12,13,16,28,29]. These earlier studies suggested that the 7-oxo bile acid reductase is a microsomal enzyme preferentially using NADPH [16]. To identify this 7-oxo bile acid reductase, we first incubated human liver microsomes with 7-oxoLCA and studied the properties of the enzymatic reaction. After incubation for 40 min, approximately 70% of 7-oxoLCA was converted, mainly into CDCA and into approximately three times lower amounts of UDCA (Figure 2A). In addition, some minor products, including muricholic acids, were observed, but were not analysed further. The 7-oxoLCA reduction was approximately ten times more efficient in the
presence of NADPH compared with NADH, and no 7-oxoLCA formation could be detected when microsomes were incubated with CDCA or UDCA and either NADP⁺ or NAD⁺ respectively, suggesting that the enzyme acts exclusively as a reductase.

Ketoconazole (5 μM) had no effect either on the amount of CDCA and UDCA formed or on their ratio, suggesting that CYPs play a minor role in the metabolism of 7-oxoLCA to its 7-hydroxylated forms. Experiments using the detergent Nonidet P40 suggested latency of the 7-oxo bile acid reductase; however, prolonged incubation with the detergent also seemed to inhibit the enzyme activity (results not shown).

In the ER lumen, NADPH is regenerated by H6PDH, which, under physiological conditions, is primarily dependent on G6P [30]. We therefore tested whether the 7-oxo bile acid reductase is stimulated in the presence of the hexose phosphate. In the presence of G6P, the 7-oxoLCA supplied was almost completely metabolized. Comparable stimulation was observed in the presence of G6S (Figure 2A), which is a specific substrate of the luminal H6PDH, but not the cytoplasmic G6PDH [30]. Furthermore, the glucose-6-phosphate translocase inhibitor S3483 abolished the G6P- and G6S-induced stimulation of 7-oxoLCA reduction (results not shown).

To our knowledge, the only currently known luminal oxidoreductase using NADPH is 11β-HSD1, which is a reversible enzyme and catalyses the interconversion of glucocorticoids and some other substrates, including 7-oxysterol, 7-oxocholesterol, 7-oxocholesterol, 7-oxocholesterol and 11-oxoandrogen metabolites [22,23–33]. An antibody raised against human 11β-HSD1 detected a single band at approximately 35 kDa and confirmed the high expression in human liver microsomes [34–36] (Figure 2B). A band at approximately 33 kDa was detected in rat liver microsomal preparations. The size difference can be explained by the presence of three glycosylation sites in human 11β-HSD1 and two in the rat enzyme [37]. The occurrence of three non-specific bands in rat liver microsomes and in HEK-293 microsomal preparations indicates some cross-reactivity of the antibody. The recombinant enzyme was constructed with a C-terminal FLAG epitope, resulting in a slightly slower migration of the protein in gel electrophoresis. Probing the blot with anti-FLAG antibody resulted in a single band at 35 kDa (results not shown).

To test whether 11β-HSD1 might catalyse the reduction of 7-oxoLCA, we used human liver microsomes and studied the effect of three structurally unrelated 11β-HSD1 inhibitors, i.e. glycyrrhetinic acid, T0504 (also known as Merck-544) and BNW16 [25]. All three inhibitors abolished the conversion of 7-oxoLCA into CDCA and UDCA (Figure 2A). Next, we compared the reduction of 7-oxoLCA and cortisone. The human liver microsomes (0.2 mg in a reaction volume of 500 μl) converted approximately 50 and 80% of 7-oxoLCA (1 μM) after 10 and 20 min respectively, and 7-oxoLCA was almost completely metabolized after 40 min (Figure 3). In comparison, in analogous experiments, 37, 56 and 67% of cortisone was converted, indicating a higher capacity to metabolize 7-oxoLCA compared with cortisone.

**Reduction of 7-oxoLCA by recombinant 11β-HSD1**

To verify that the reduction of 7-oxoLCA indeed is catalysed by 11β-HSD1, experiments in lysates of HEK-293 cells expressing the recombinant enzyme were performed. 11β-HSD1 efficiently catalysed the reduction of 7-oxoLCA with an apparent Keq of 980 ± 210 nM and a Vmax of 2.8 ± 0.4 nmol·mg⁻¹·h⁻¹ as calculated by four-parametric non-linear regression (Figure 4A). Comparable values were obtained using the Hanes–Woolf equation (Figure 4B). No conversion of 7-oxoLCA was observed in untransfected HEK-293 control cells. The taurine- and glycine-conjugated 7-oxo bile acids, 7-oxoLC-Tau and 7-oxoLC-Gly, were similarly converted into the 7-hydroxylated CDC-Tau (chenodeoxycholyltaurine) and CDC-Gly (chenodeoxycholylglycine) with minor amounts of UDC-Tau (ursodeoxycholyltaurine) and UDC-Gly (ursodeoxycholylglycine) respectively, demonstrating that 11β-HSD1 accepts both unconjugated and conjugated 7-oxoLCA as substrate.

Next, we studied the impact of H6PDH on 11β-HSD1-dependent reduction of 7-oxoLCA in intact HEK-293 cells stably expressing either human 11β-HSD1 alone or co-expressing 11β-HSD1 and H6PDH. Co-expression of H6PDH stimulated the 7-oxo reductase activity of 11β-HSD1 (Figure 5). In cells co-expressing 11β-HSD1 and H6PDH, the reaction was almost completed after 24 h, resulting in the formation of approximately 90% CDCA and 10% UDCA. In contrast, only approximately 50% of 7-oxoLCA was converted in cells expressing solely 11β-HSD1, and it took more than 48 h until the reaction was completed (results not shown). No oxidation of CDCA and UDCA was detected, independent of the cell line used, confirming the observation from human liver microsomes and showing that 11β-HSD1 catalyses the irreversible conversion of 7-oxoLCA into CDCA and lower amounts of UDCA.

**Interference of bile acids with the metabolism of glucocorticoids by 11β-HSD1**

Several bile acids, including CDCA and LCA, have been found in previous studies to inhibit 11β-HSD1 and 11β-HSD2 respectively.
7-Oxolithocholic acid is reduced by 11β-HSD1

Enzymatic activity of human 11β-HSD1 was determined in lysates of transfected HEK-293 cells as described in the Experimental section. (A) Reaction rate against substrate concentration plot of 10 min incubations with 7-oxoLCA. (B) Hanes–Woolf plot of the data shown in (A). Results (n = 3) are means ± S.D.

Figure 4 Enzyme kinetic profile of 7-oxo reduction of 7-oxoLCA by recombinant human 11β-HSD1

Enzymatic activity of human 11β-HSD1 was determined in lysates of transfected HEK-293 cells as described in the Experimental section. (A) Reaction rate against substrate concentration plot of 10 min incubations with 7-oxoLCA. (B) Hanes–Woolf plot of the data shown in (A). Results (n = 3) are means ± S.D.

Figure 5 Impact of H6PDH on 11β-HSD1-dependent 7-oxo reduction of 7-oxoLCA in living cells

Intact HEK-293 cells stably expressing human 11β-HSD1 (A) or 11β-HSD1 and H6PDH (B) were incubated for 0–24 h with 1 μM 7-oxoLCA, followed by quantification of bile acids by LC–MS. Results (n = 3) are means ± S.D.

Table 1 Inhibition by bile acids of the oxidation and reduction of glucocorticoids by 11β-HSD1

11β-HSD activities were measured in lysates of HEK-293 cells expressing the respective human recombinant enzyme as described in the Experimental section. Results are IC₅₀ values in μM (means ± S.D.) from four independent experiments.

<table>
<thead>
<tr>
<th>Bile acid</th>
<th>11β-HSD1 oxidation IC₅₀ (μM)</th>
<th>11β-HSD1 reduction IC₅₀ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-OxoLCA</td>
<td>2.8 ± 0.8</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>LCA</td>
<td>0.21 ± 0.06</td>
<td>0.36 ± 0.05</td>
</tr>
<tr>
<td>CDCA</td>
<td>0.27 ± 0.08</td>
<td>4.1 ± 0.6</td>
</tr>
<tr>
<td>UDCA</td>
<td>2.3 ± 1.0</td>
<td>6.9 ± 1.2</td>
</tr>
<tr>
<td>DCA</td>
<td>7.2 ± 2.0</td>
<td>8.9 ± 1.4</td>
</tr>
<tr>
<td>CA</td>
<td>&gt;50</td>
<td>50 ± 13</td>
</tr>
<tr>
<td>7-OxoLC-Tau</td>
<td>&gt;50</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>7-OxoLC-Gly</td>
<td>&gt;50</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>CDC-Tau</td>
<td>0.61 ± 0.05</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>CDC-Gly</td>
<td>1.0 ± 0.2</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>UDC-Tau</td>
<td>23 ± 6</td>
<td>&gt;50</td>
</tr>
<tr>
<td>UDC-Gly</td>
<td>25 ± 5</td>
<td>&gt;50</td>
</tr>
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</table>

We therefore compared the effect on 11β-HSD1 activities of 7-oxoLCA and its taurine- and glycine-conjugated forms with that of other relevant bile acids (Table 1). Whereas the 7α-hydroxylated CDCA and its conjugated derivatives CDC-Tau and CDC-Gly showed a more than 10-fold preference to inhibit the dehydrogenase over the reductase activity of 11β-HSD1, 7-oxoLCA displayed a slight preference to inhibit the reduction of cortisone, an effect that was more pronounced for the conjugated derivatives. The 7β-hydroxylated bile acids UDCA and UDC-Tau preferentially inhibited 11β-HSD1 dehydrogenase activity; however, they were approximately 10-fold less potent than the 7α-hydroxylated forms.

Impact of 7-oxoLCA on the ratio of cortisol to cortisone at steady state

Bile acids can reach high concentrations in the liver and may affect not only initial rates of conversion, but also steady-state ratios of cortisol to cortisone controlled by 11β-HSD1. We therefore determined the effect of 7-oxoLCA on the steady-state ratio of cortisol to cortisone in HEK-293 cells stably expressing 11β-HSD1 or co-expressing 11β-HSD1 and H6PDH. As shown in Figure 6(A), approximately 40% cortisol was produced in 11β-HSD1-expressing cells, whereas over 90% of initially supplied cortisone was converted into cortisol upon co-expression with H6PDH, in line with earlier observations [33]. A mirror image was obtained when cells were incubated initially with cortisol (Figure 6B). Co-incubation of the cells with the respective glucocorticoid and increasing concentrations of 7-oxoLCA resulted in diminished cortisol production when cortisone was supplied and enhanced cortisone formation when cortisol was supplied initially, thus reflecting a shift from the active to the inactive glucocorticoid at steady state in the presence of high concentrations of 7-oxoLCA.

Analysis of binding of bile acids to 11β-HSD1 by three-dimensional modelling

We used the crystal structure of 11β-HSD1 with corticosterone [26] (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/436/bj4360621add.htm) as a benchmark for comparison of the three-dimensional models of 11β-HSD1 with 7-oxoLCA, CDCA and UDCA (Figure 7). In the crystal structure of 11β-HSD1 in complex with corticosterone, the phenolic group of Tyr⁸³ on 11β-HSD1 and C4 on NADP⁺ are 2.8 and 3.9 Å (1 Å = 0.1 nm) respectively from the 11β-hydroxy group on
Figure 6  Effect of 7-oxoLCA on the steady-state ratio of cortisol to cortisone

(A) Intact HEK-293 cells expressing 11β-HSD1 (white bars) or co-expressing 11β-HSD1 and H6PDH (black bars) were incubated for 24 h with 200 nM radiolabelled cortisone and increasing concentrations of 7-oxoLCA, followed by determination of cortisol formation. (B) Similarly, cells were incubated with 200 nM radiolabelled cortisol, followed by measuring cortisone formation. Results representative of three independently performed experiments are shown.

Figure 7  Three-dimensional models of mouse 11β-HSD1 complexed with CDCA, 7-oxoLCA and UDCA

In the complexes of 11β-HSD1 with CDCA and 7-oxoLCA, Tyr^{183} and C-4 on NADP(H) have favourable contacts with the 7α-hydroxy group on CDCA and 7-carbonyl on 7-oxoLCA. The phenolic hydroxy group on Tyr^{183} on 11β-HSD1 is 4.1 Å from the 7β-hydroxy group on UDCA. The ε-amino group on Lys^{187} has stabilizing interactions with the ribose hydroxy groups on NADPH for 11β-HSD1 complexed with 7-oxoLCA. However, this is not the case with NADP^{+} and 11β-HSD1 complexed with CDCA and UDCA. Arg^{66} has an electrostatic interaction with the 2′-phosphate on NADP(H) in all three complexes.

Although all three bile acid substrates have favourable interactions with 11β-HSD1, there are differences in some interactions that can explain differences in the observed binding and catalytic activity. In the three-dimensional model of 11β-HSD1 in complex with 7-oxoLCA, the phenolic group on Tyr^{183} on 11β-HSD1 and C-4 on the nicotinamide ring of NADPH are 2.9 and 3.5 Å respectively from the 7-oxo group on 7-oxoLCA. The ε-amino group on Lys^{187} has two favourable contacts with the 2′- and 3′-ribose hydroxy groups on NADPH, and the hydroxy group on Tyr^{183} is hydrogen-bonded with the 2′-hydroxy group on the nicotinamide ribose. The ε-amino group on Lys^{187} is 3.2 Å from the 2′- and 3′-hydroxy groups on the nicotinamide ribose. This stabilizes the orientation of the nicotinamide ribose [26]. Nε and Nη on the guanidinium group of Arg^{66} have an electrostatic interaction with the 2′-phosphate of NADP^{+}. This electrostatic interaction is characteristic of NADP(H)-dependent SDRs (short-chain dehydrogenase/reductases), including 17β-HSD1 [41–43].
on Tyr$^{183}$ is 3.5 Å from the 2'-hydroxy group of the nicotinamidimido ribose. Furthermore, Arg$^{66}$ and other residues in the N-terminal end of 11β-HSD1 have favourable contacts with the adenosine on NADPH. Together, this three-dimensional model indicates that 7-oxoLCA fits into 11β-HSD1 in an orientation that favours reduction of the 7-oxo group to an alcohol.

In the three-dimensional model of 11β-HSD1 in complex with CDCA, Tyr$^{183}$ on 11β-HSD1 and C-4 on NADP$^+$ are 2.9 and 3.4 Å respectively from the 7α-hydroxy group on CDCA. However, Lys$^{187}$ has an asymmetric orientation to the ribose hydroxy groups. The ε-amino group on Lys$^{187}$ is 4.6 Å from 3'-hydroxy group, which is too far to form a hydrogen bond. Also, the hydroxy group on Tyr$^{183}$ is 4.4 Å from the 2'-hydroxy group of the nicotinamidimido ribose. Thus the nicotinamidimido ribose lacks two stabilizing interactions that are present in 11β-HSD1 in complex with 7-oxoLCA. Arg$^{66}$ and other residues in the N-terminal end of 11β-HSD1 have favourable contacts with the adenosine on NADP$^+$.

In the three-dimensional model of 11β-HSD1 in complex with UDCA, Tyr$^{183}$ on 11β-HSD1 and C-4 on NADP$^+$ are 4.1 and 3.3 Å respectively from the 7β-hydroxy group on UDCA. Lys$^{187}$ has an asymmetric orientation to the ribose hydroxy groups. The ε-amino group on Lys$^{187}$ is 4.5 Å from 3'-hydroxy group, which is too far to form a hydrogen bond. Arg$^{66}$ and other residues in the N-terminal end of 11β-HSD1 have favourable contacts with the adenosine on NADP$^+$ (see also the Supplementary Online Data).

We also constructed three-dimensional models of 11β-HSD1 in complex with glycine conjugates of 7-oxoLCA, CDCA and UDCA, as shown in Supplementary Figure S2 at http://www.BiochemJ.org/bj/436/bj4360621add.htm. All three glycine–bile acid conjugates have stabilizing contacts with various backbone nitrogens or oxygens in 11β-HSD1. Examination of these three-dimensional models reveals an unexpected coulombic interaction between the glycine carboxyl group and Nε2 on Arg$^{66}$, which also has a key electrostatic interaction with the 2'-ribose phosphate on NADP$^+$. Thus Arg$^{66}$ has two important stabilizing interactions in the complexes of 11β-HSD1 with glycine conjugates of bile acids.

Thus the three-dimensional models of 11β-HSD1 with bile acids (Figure 7) reveal that 7-oxoLCA has the most favourable interaction with the catalytic site in 11β-HSD1 and that glycine–bile acid conjugates also can fit into 11β-HSD1 in which they have a coulombic interaction with Arg$^{66}$, a key residue in the stabilization of NADPH binding to 11β-HSD1.

**DISCUSSION**

By catalysing the biotransformation of gut bacteria-derived secondary bile acids, the liver plays a key role in damage and repair; damage being changes in the steroid nucleus by bacterial enzymes, and repair being rectification of these changes by the hepatocyte. Several bacterial strains express 7α-HSIDs to yield 7-oxoLCA from CDCA and 7-oxoDCA from CA [7]. 7-OxoLCA can be metabolized further by reversible bacterial 7β-HSIDs, or taken up actively via sodium-dependent transporter [SLC10A2 (solute carrier 10A2)] from the lumen of the ileal segment or passively in the colon. Although 7-oxoLCA is readily detectable in faeces and portal blood, it cannot be detected at substantial levels in bile and plasma [44, 45], suggesting efficient hepatic metabolism.

In the present paper, we report the identification of 11β-HSD1 as a hepatic 7-oxoreductase, providing an explanation for the low circulating 7-oxoLCA concentrations. Human and rodent liver expresses high levels of 11β-HSD1 [34, 35] (Figure 2B). Previous studies demonstrated that 11β-HSD1 purified from human or rodent liver catalyses the NADPH-dependent conversion of cortisone into cortisol and 11-dehydrocorticosterone to corticosterone respectively [36]. In addition, it was shown that 11β-HSD1 purified from rabbit and hamster liver accepts not only glucocorticoids as substrates, but also 7-oxocholesterol metabolites [46, 47]. We now demonstrate that both human liver microsomes and recombinant human 11β-HSD1 expressed in HEK-293 cells catalyse the NADPH-dependent 7-oxo reduction of 7-oxoLCA to form preferentially the 7α-hydroxy bile acid CDCA and to a lesser extent the 7β-hydroxy isomer UDCA (10–20%). The ratio of CDCA to UDCA observed in our experiments with human liver microsomes as well as recombinant human enzyme is in line with earlier observations with human liver preparations and measurements in blood following i.v. administration [13, 16]. Importantly, the 7-oxo reduction of 7-oxoLCA in liver microsomes was completely abolished by the 11β-HSD1 inhibitors glycyrrhetinic acid, T0504 and BNW16 (Figure 2A). Although we cannot exclude the existence of another enzyme that catalyses the 7-oxo reduction of 7-oxoLCA in the liver, it is highly unlikely that such an enzyme would be completely inhibited by all of the three structurally unrelated compounds. Thus the results provide strong evidence that 11β-HSD1 is the major enzyme catalysing the 7-oxo reduction of 7-oxoLCA in humans.

Analysis of the kinetic properties revealed that 11β-HSD1 efficiently catalyses 7-oxo reduction of 7-oxoLCA with approximately 2-fold lower affinity, but 2-fold higher $V_{\text{max}}$ compared with reduction of cortisone. The conversion of both 7-oxoLCA and cortisone was latent, dependent on H6PDH and stimulated to a similar extent by addition of G6P to the reaction mixture. 11β-HSD1 accepted the taurine- and glycine-conjugated forms as substrates, with catalytic efficiencies comparable with those for the free bile acids. This is consistent with predictions of the three-dimensional models of the three bile acids conjugated to glycine, which uncovered an unexpected interaction between the glycine carboxyl group and Nε2 on Arg$^{66}$, which also has a key electrostatic interaction with the 2'-ribose phosphate on NADP$^+$. Thus Arg$^{66}$ has two important stabilizing interactions in the complexes of 11β-HSD1 with glycine conjugates of bile acids.

Unlike other steroid and sterol substrates, 11β-HSD1 irreversibly catalyses the 7-oxo reduction of 7-oxoLCA, and the stereoselectivity for the bile acid metabolites formed is just opposite of that observed for the metabolites of 7-oxocholesterol [31, 32], 7-oxodehydroepiandrosterone and 7-oxopregnenolone [33]. Neither CDCA nor UDCA, even upon prolonged incubation and at high concentrations, were converted into 7-oxoLCA, and there was also no isomerization of CDCA to UDCA or vice versa, as has been observed for 7α- and 7β-hydroxyepiandrosterone [48] and 7α- and 7β-hydroxydehydroepiandrosterone [33] respectively. Our results are in line with an earlier report on the metabolism of radiolabelled CDCA in rats with bile fistulas [12], where conversion of CDCA into trihydroxylated metabolites and minor amounts of UDCA, but no formation of 7-oxoLCA was observed. Furthermore, in humans, after a single hepatic passage following i.v. administration, neither CDCA nor UDCA was modified on the steroid ring.

The three-dimensional models indicate that only 7-oxoLCA has optimal binding of substrate and cofactor to Tyr$^{183}$ and Lys$^{187}$ (Figure 7), which is necessary for reduction of 7-oxoLCA to CDCA. In contrast, in the three-dimensional models of 11β-HSD1 with CDCA and UDCA, the ε-amino group on the key catalytic
residue Lys187 is too far from the 3′-ribose hydroxy group on NADP+ to form a stabilizing hydrogen bond required for catalytic activity. The distance between the 7β-hydroxy group on UDCA and the phenolic group on Tyr183 is 4.1 Å, which indicates weaker binding than found for the similar interaction between Tyr183 and either CDCA or 7-oxoLCA. This may explain the preference for binding than found for the similar interaction between Tyr183 and activity. The distance between the 7
3.

Alterations in the availability of bile acids, which reach high concentrations in the hepatocyte in cholestatic liver disease, may affect the hepatic activation of glucocorticoids. In intact HEK-293 cells expressing 11β-HSD1, but not H6PDH, CDCA and its conjugates preferentially inhibited 11β-HSD1 dehydrogenase activity and stimulated cortisone reduction. However, upon co-expression with H6PDH, which reflects the situation in hepatocytes, CDCA displayed weak inhibitory activity on 11β-HSD1. In contrast, 7-oxoLCA preferentially inhibited 11β-HSD1 reductase activity, and the presence of high concentrations of 7-oxoLCA stimulated cortisol oxidation and shifted the ratio of active to inactive glucocorticoids under steady-state conditions, probably by altering the ratio of NADPH to NADP+ in the ER lumen. It was shown previously that a ratio of NADPH to NADP+ greater than 10 is required for 11β-HSD1 to efficiently reduce cortisone [49]. The presence of high concentrations of 7-oxo bile acids, 7-oxo cholesterol or 7-oxo steroids may thus result in decreased ER luminal NADPH levels and lower concentrations of active glucocorticoids, thereby modulating redox signalling pathways and glucocorticoid-dependent adaptive responses.

Further research is needed to elucidate the physiological role for the rapid hepatic removal of 7-oxoLCA. Distinct effects of 7-oxoLCA, CDCA and UDCA on bile acid sensing receptors may affect the regulation of genes involved in lipid metabolism and inflammation. CDCA has been found to be a potent activator of the FXR (farnesoid X receptor)/RXR (retinoid X receptor), VDR (vitamin D receptor) and PXR (pregnane X receptor), LXR (liver X receptor) and CAR (constitutive androstane receptor) [62]. Also, there are currently no data available on potential effects of 7-oxoLCA on other nuclear receptors, including LXR (liver X receptor), VDR (vitamin D receptor) and PXR (pregnane X receptor).

Nevertheless, the results may be relevant regarding the current development of 11β-HSD1 inhibitors for treatment of metabolic diseases [19–21]. Inhibition of 11β-HSD1 is expected to abolish hepatic metabolism of 7-oxoLCA, thereby leading to elevated hepatic and circulating 7-oxoLCA levels, similar to the observed accumulation of 7-oxocholesterol following 11β-HSD1 inhibition in rats [32]. 11β-HSD1 inhibition is not expected to affect bacterially derived production of UDCA and its metabolism in the liver; however, as shown in Figure 5, some UDCA is formed in the 11β-HSD1-dependent reduction of 7-oxoLCA, and inhibition of the enzyme might lower the local availability of UDCA. Clearly, further studies in 11β-HSD1-knockout mice and pre-clinical and clinical studies using selective inhibitors are needed to elucidate the impact of 11β-HSD1 on bile acid composition and function.

AUTHOR CONTRIBUTION

Alex Odermatt had the responsibility for the overall planning and conduct of the work, performed inhibitor experiments, analysed data and wrote the paper. Thierry Da Cunha and Carlos Penno developed the LC–MS protocol, performed enzyme activity experiments and analysed data. Christian Reichert performed activity experiments. Min Dong and Armin Wolf assisted in the design of experiments with human liver microsomes and analysis of protein expression. Charlie Chandrawangshuwan performed three-dimensional modelling and analysed data, and Michael Baker performed three-dimensional modelling, analysed data and wrote the paper. All authors read and approved the final paper.

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SUPPLEMENTARY ONLINE DATA

Hepatic reduction of the secondary bile acid 7-oxolithocholic acid is mediated by 11β-hydroxysteroid dehydrogenase 1

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EXPERIMENTAL

Materials

Human liver microsomes (InVitro CYP H-class microsomes from a male donor) were obtained from Celsis International, [1,2,6,7–3H]cortisone was from American Radiolabeled Chemicals, [1,2,6,7–3H]cortisol was from GE Healthcare, 7H-1, 2,4-triazolo(4,3-a)azepine,6,7,8,9-tetrahydro-3-tricyclo(3·3·1·13·7)dec-1-yl (T0504) was from Enamine, steroids and bile acids were from Steraloids, and [2,2,4,4–3H]CDCA (>98% isotopic purity) was from Isotec, Sigma–Aldrich. Cell culture media were purchased from Invitrogen and Sigma. All other chemicals were from Fluka AG of the highest grade available. The conjugated bile acids 7-oxoLC-Tau and 7-oxoLC-Gly were a gift from Dr Alan F. Hofmann (University of California, San Diego, San Diego, CA, U.S.A.) [1]. BNW16 was provided by Dr Thomas Wilckens (BioNetWorks, Munich, Germany) [2], and S3483 was obtained from Sanofi-Aventis. Chemicals were diluted from 10 mM stock solutions in DMSO or methanol using TS2 buffer (final solvent concentrations were kept below 0.2 %).

Measurement of the interconversion of glucocorticoids in cell lysates

HEK-293 cells stably expressing recombinant human 11β-HSD1 alone or co-expressing 11β-HSD1 and H6PDH (AT6 and HHH7 clones respectively [3]) were cultured in DMEM supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine. For measurements with cell lysates, cells were detached and centrifuged, and pellets were stored at −80°C. Cell pellets were resuspended in TS2 buffer (100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM MgCl2, 250 mM sucrose and 20 mM Tris/HCl, pH 7.4), sonicated and used immediately to measure enzyme activity. Lysates of HEK-293 cells expressing human 11β-HSD1 were incubated for 10 min at 37°C in a total volume of 22 μl containing 200 nM and 10 nCi of [1,2–3H]cortisone or [1,2,6,7–3H]cortisol and 500 μM cofactor NADPH or NADP+ respectively and vehicle or various concentrations of bile acids. Following conversion of radiolabelled glucocorticoids and termination of reactions by adding methanol containing 2 mM unlabelled cortisone and cortisol, 15 μl was spotted on Polygram SIL G-25 UV254 silica plates (Macherey-Nagel), plates were dried, and cortisone and cortisol were separated using a solvent system of 9:1 (v/v) chloroform/methanol. The separated steroids were analysed by scintillation counting.

Results (means ± S.D.) were obtained from at least three independent experiments. Enzyme kinetics was analysed by non-linear regression using four-parameter logistic curve fitting. For statistical comparisons, the ratio t-test in GraphPad Prism 5 software was used.

Analysis of non-labelled steroids and bile acids by LC–MS/MS

Frozen samples from reactions using intact cells, cell lysates or microsomes were thawed, and a fixed amount of deuterated CDCA (0.5 nmol) or corticosterone (0.2 nmol) was added as an internal standard, followed by mixing and centrifugation at 3000 g for 5 min. Supernatants were loaded on to Oasis HLB SPE cartridges (pre-conditioned with 1 ml of methanol and 1 ml of water), followed by washing with 2 ml of water and elution with 2 ml of methanol. The solvent was evaporated and the residue reconstituted in 100 μl of methanol.

7-OxoLCA and its metabolites were separated on an Atlantis T3 (3 μm, 2.1 mm × 150 mm) column (Waters) at 30°C using an Agilent Technologies model 1200 liquid chromatograph. The mobile phase consisted of solvent A (0.1 % formic acid in water) and solvent B (0.1 % formic acid in acetonitrile). A linear gradient was used starting from 65 % solvent A and 35 % solvent B to 5 % solvent A and 95 % solvent B from 0 to 10 min, then 5 % solvent A and 95 % solvent B from 10 to 12 min, 5 % solvent A and 95 % solvent B to 65 % solvent A and 35 % solvent B from 12 to 13 min, and finally re-equilibration with 65 % solvent A and 35 % solvent B from 13 to 18 min. The flow rate was maintained at 0.4 ml/min. The LC was interfaced to an Agilent 6410 triple quad mass spectrometer. The injection volume of each sample was 5 μl. The mass spectrometer was operated in atmospheric pressure electrospray positive-ionization mode, with a source temperature of 350°C, a gas flow of 10 l/min and nebulizer gas pressure of 45 psi (1 psi = 6.9 kPa) at capillary and cone voltages of 4 kV and 190 V respectively. Data acquisition was performed using MassHunter workstation software (version B.01.04).

For the separation of cortisone and cortisol, a linear gradient was used starting from 70 % solvent A and 30 % solvent B to 5 % solvent A and 95 % solvent B from 0 to 13 min, then 5 % solvent A from 13 to 15 min, 5 % solvent A and 95 % solvent B to 70 % solvent A and 30 % solvent B from 15 to 18 min and finally re-equilibration with 70 % solvent A and 30 % solvent B for 5 min. The flow rate was maintained at 0.3 ml/min using the same MS conditions as for bile acids.

Bile acid and glucocorticoid metabolites were identified and quantified as outlined in the main text.

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RESULTS

Analysis of binding of bile acids to 11β-HSD1 by three-dimensional modelling

Figure S1 shows a three-dimensional model based on the crystal structure of 11β-HSD1 with corticosterone. In addition to the main stabilizing interactions described in the main text, the three-dimensional models also reveal differences in the stabilization of the A-ring on the three bile acids and corticosterone by 11β-HSD1 (compare Figure S1 with Figure 7 of the main text). All three bile acids have different interactions compared with corticosterone. Gln177 on 11β-HSD1 has a stabilizing contact with the C-3 hydroxy group on each bile acid. Ala172 also stabilizes the C-3 hydroxy group on CDCA and 7-oxoLCA. Pro178 has a contact with the C-3 hydroxy group on UDCA that is not found in CDCA and 7-oxoLCA. None of these residues has a stabilizing contact with C-3 ketone on corticosterone, which has a van der Waals contact with the backbone nitrogen on Leu137 [4].

Interestingly, the crystal structure of human 11β-HSD1 complexed with CHAPS as determined by Hosfield et al. [5] has the 7α-hydroxy group on CHAPS approximately 2.93 Å from Tyr183, which could mean that 11β-HSD1 metabolizes this bile acid. However, regarding turnover of CHAPS or its core CA by 11β-HSD1, our analysis indicates that 11β-HSD1 does not oxidize 7α-hydroxy bile acids. Indeed, 11β-HSD1 preferentially metabolizes 7-oxoLCA.

Figure S1  Binding of corticosterone to mouse 11β-HSD1

Tyr183 and Lys187 have critical stabilizing contacts respectively with the 11β-hydroxy group on corticosterone and ribose hydroxy groups on NADP⁺. The guanidinium group on Arg66 forms a salt bridge with the 2′-phosphate on NADP⁺.
7-Oxolithocholic acid is reduced by 11β-HSD1

Figure S2 Three-dimensional models of mouse 11β-HSD1 complexed with glycine conjugates of CDCA, 7-oxoLCA and UDCA

In all three three-dimensional models, the carbonyl group on each glycine–bile acid conjugate has a stabilizing interaction with the guanidinium group of Arg<sup>66</sup>. Backbone interactions also stabilize the glycine substituent.

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