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A Novel NADP⁺- Dependent Dehydrogenase Activity for 7 α / β and 11 β -hydroxysteroids in human liver nuclei: A Third 11 β -Hydroxysteroid Dehydrogenase

B. Robinson¹ and R.A. Prough²

¹Department of Animal Science, The Robert H. Smith Faculty of Agricultural, Food and Environment, The Hebrew University of Jerusalem, P.O.B. 12, Rehovot 76100, Israel

²Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, KY 40292, U.S.A. Email: russ.prough@louisville.edu

Abstract

Human tissue from uninvolved liver of cancer patients was fractionated using differential centrifugation and characterized for 11 β HSD enzyme activity against corticosterone, dehydrocorticosterone, 7 α and 7 β -hydroxy-dehydroepiandrosterone, and 7-oxodehydroepiandrosterone. An enzyme activity was observed in nuclear protein fractions that utilized either NADP⁺ or NAD⁺, but not NADPH and NADH, as pyridine nucleotide cofactor with K_m values of 12 ± 2 and 390 ± 2 μ M, compared to the K_m for microsomal 11 β HSD1 of 43 ± 8 and 264 ± 24 μ M, respectively. The K_m for corticosterone in the NADP⁺-dependent nuclear oxidation reaction was 102 ± 16 nM, compared to 4.3 ± 0.8 μ M for 11 β HSD1. The K_{cat} values for nuclear activity with NADP⁺ was 1,687 nmol/min/mg/ μ mol, compared to 755 nmol/min/mg/ μ mol for microsomal 11 β HSD1 activity. Inhibitors of 11 β HSD1 decreased both nuclear and microsomal enzyme activities, suggesting that the nuclear activity may be due to an enzyme similar to 11 β HSD Type 1 and 2.

Keywords

Corticosterone; dehydrocorticosterone; dehydroepiandrosterone; DHEA; 7 α -hydroxy-DHEA; 7- β -hydroxyDHEA; 7-oxo-DHEA; 11 β -hydroxysteroid dehydrogenase; liver; human

INTRODUCTION

The metabolism of glucocorticoids (GC) and 7-hydroxylated sterols has been shown to be dependent on several isozymes of the 11 β -hydroxysteroid dehydrogenase (11 β HSDs) family and as they have common substrates (corticosterone, 7 α -hydroxy-cholesterol, and 7 α hydroxy-dehydroepiandrosterone), these compounds may inhibit their respective metabolism. 11 β HSDs are membrane-bound, pyridine nucleotide- dependent dehydrogenases found in endoplasmic reticulum and other membrane organelles [1-4]. In liver, the principal enzyme observed is the

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Phone: 972-8-9489396; Fax 972-8-9465763; Email: robinzon@agri.huji.ac.il.

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microsomal 11 β HSD Type 1 (11 β HSD1) that utilizes either NADP⁺ or NADPH to catalyze the reversible oxidation-reduction of the substrates mentioned above. The microsomal 11 β HSD1 serves as a reductase, when coupled in microsomal vesicles with hexose-6-phosphate dehydrogenase activity [5;6]. In kidney, 11 β HSD1 serves as an NADP⁺-dependent dehydrogenase, since it is apparently not coupled with hexose-6-phosphate dehydrogenase in this tissue. Other isozymes of the 11 β HSD super-family 11 β HSD super-family (11 β HSD2 and 3) catalyze unidirectional reactions that are reported to be dependent upon either NAD⁺ or NADP⁺, respectively, as oxidized pyridine nucleotide cofactors to oxidize GC from their active alcohol form (cortisol and corticosterone) to the in-active oxo- or keto-form (cortisone and dehydrocorticosterone) in an irreversible reaction [1;6-8]. In human kidney nuclei, immunoreactive human 11 β HSD2 accounted for 40% of total cellular 11 β HSD2 protein content [1]. In kidney from rat and sheep, a third dehydrogenase (11 β HSD3) was shown to be present in membranes of Golgi apparatus and mitochondria [7], while 11 β HSD2 is preferentially located in the nuclear membrane [6]. Because of their differing sub-cellular organelle distribution and function in GC metabolism, 11 β HSD activity possibly prevent excess active GC in plasma from binding to nuclear MR. However, the NADPH-dependent 11 β HSD1 activity would allow formation of bioactive GC, thereby serving as a pre-receptor regulated process in other tissues.

We previously demonstrated that hamster, pig and rat liver microsomal fractions served as oxidoreductases for both GC and 7-oxidized-DHEA metabolites [9-12]. All three species contained significant microsomal 7 α - and 7 β -hydroxy-DHEA (7 α - and 7 β -OH-DHEA) to 7-oxo-DHEA and reducing 7-oxo-DHEA to 7 α -OH-DHEA. Recently, we observed an NAD⁺-dependent, unidirectional 11 β HSD that oxidizes corticosterone (CS) into dehydrocorticosterone (DHC) and 7 α -OH-DHEA into 7-oxo-DHEA in the nuclear fraction of rat and pig kidney; however, it does not catalyze the reduction of DHC or 7-oxo-DHEA [11; 12]. In the pig liver nuclei, we found a high-affinity, low-capacity, NADP⁺-dependent, unidirectional 11 β HSD that oxidizes, and therefore, inactivates GC action (Robinson and Prough, unpublished results). In the current study, we also observed another NADP⁺-dependent, unidirectional 11 β HSD activity in human liver nuclear protein fractions. This nuclear 11 β HSD is of high-affinity and low-capacity (K_m of 102 ± 16 nM for dehydrocorticosterone and V_{max} of 172 ± 42 pmol product produced/min/mg protein), similar to the rate previously reported for 11 β HSD2 and 3 in kidney [7;11;13]. Because this enzyme has a much higher affinity for NADP⁺ than for NAD⁺ (K_m for the co-substrate of 12 ± 2 μ M vs. 390 ± 43 μ M, respectively), it is similar to the putative 11 β HSD3 found in rat, pig and sheep kidney. We have characterized the metabolism of GC and 7-oxidized-DHEA derivatives by freshly prepared human liver microsomal and nuclear protein fractions, their co-substrate dependency and response to specific 11 β HSDs inhibitors. The presence of an NADP⁺-dependent 11 β -HSD activity in the nuclei fractions of human liver is distinctly different than the enzymes previously described in the rodent or pig [12]. Therefore, we suggest that the nuclear membrane of the human hepatocyte is equipped with an 11 β HSD3-like enzyme that prevents *in situ* activated GC from penetrating the nucleus and altering gene expression. This enzyme activity may protect human hepatic genome from glucocorticoid excess caused by high 11 β -HSD1 activity, possibly due to the presence of the putative 11 β HSD3.

MATERIALS AND METHODS

Chemicals

DHEA, 7 α -OH-DHEA, 7 β -OH-DHEA and 7-oxo-DHEA, 11 α -hydroxyprogesterone (11 β -OH-PRO), 11 β -hydroxy-progesterone (11 β -OH-PRO), CS and DHC were obtained from Steraloids Inc. (Newport, RI). Carbenoxolone (CBX), β -NADPH, β -NADP⁺, β -NAD⁺ and β -NADH were purchased from Sigma Chemical Co., Inc., (St. Louis, MO, USA). HPLC grade hexane, ethyl acetate, ethanol, chloroform and acetone were purchased from Fisher Scientific

Co. (Pittsburgh, PA, USA). [1,2,6,7-³H]-DHEA and [1,2,6,7-³H]-CS were purchased from NEN Life Science Products (Boston, MA, USA). Tritiated 7 α -OH-DHEA, 7 β -OH-DHEA and 7-oxo-DHEA were prepared as described previously [14]. In brief, [1,2,6,7-³H]-DHEA was incubated with pig liver microsomal protein, NADPH-regenerating system containing 1 mM β -NADPH, 0.8 mM isocitrate, and 0.1 U/mL isocitrate dehydrogenase, and 1mM NADP⁺ for production of 7 α -OH-DHEA, 7 β -OH-DHEA and 7-oxo-DHEA. The metabolites were extracted three times with 5 mL ethyl acetate and then were isolated by preparatory TLC (Silica Gel GF 2000 μ ; Analtech; Newark, DE) using ethyl acetate:hexane:glacial acetic acid (18:8:3 V:V:V) as the mobile phase. The metabolites that co-migrated with authentic standards were extracted from the TLC media with ethyl acetate and dried under a stream of nitrogen. Aliquots from each fraction were assayed by GC-MS in a single-blind manner and documented to contain the expected 7-oxidized metabolite. Each of the metabolites was dissolved in ethanol with cold synthetic metabolite to attain a specific radioactivity of 450 μ Ci/mmol. Similarly, tritiated CS was used to prepare DHC radiotracer by incubation with pig liver microsomal protein fractions in a NADP⁺-containing medium; the metabolite was extracted with 5 ml ethyl acetate followed by 5 ml chloroform; and subsequently, the metabolites were isolated by preparatory TLC using chloroform:acetone 5:1 as the mobile phase. The tritiated CS and DHC then were dissolved in ethanol and unlabelled synthetic metabolite added to attain a specific radioactivity of 450 μ Ci/mmol. When lower concentrations of CS were used for enzyme assays, CS substrate was prepared at specific radioactivity of 5.6 mCi/mmol.

Tissues

Samples of normal human liver tissue, collected from eight patients (five females and three males, 41-78 years old), were used in this study. These samples were received from the Cooperative Human Tissue Network (CHTN, Birmingham, AL. The samples were collected during right lobe partial hepatectomy of metastatic adenocarcinoma, frozen immediately and stored at -80°C before further processing.

Preparation of subcellular fractions

The tissues were processed as described previously [12]. In brief, the liver samples were homogenized in 4 volumes (V/W) of 50 mM potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA. The homogenate was sedimented at 300 $\times$ g for 10 min to remove cellular debris. The supernatant was then sedimented at 1,000 $\times$ g for 10 min to obtain nuclear pellets that were resuspended in 0.15 M KCl, 50 mM potassium phosphate buffer, pH 7.4 (wash buffer) and sedimented a second time. The final nuclei preparation was resuspended in 50 mM potassium phosphate buffer, pH 7.4, containing 0.25 M sucrose and 1 mM EDTA, and 10% glycerol (preservation buffer) in a volume equivalent of the original tissue weight. Following removal of the nuclear pellet from the homogenate, the supernatant was sedimented in 12,000 $\times$ g for 20 min followed by 18,000 $\times$ g for another 20 min. The resulting supernatant then was sedimented at 108,000 $\times$ g for 60 min to obtain the microsomal pellet. This pellet was resuspended in the wash buffer, sedimented a second time under the same conditions and resuspended in preservation buffer at the volume equal to the original tissue weight. All fractions were stored at -80°C pending the metabolic assays. Protein concentration was determined by measuring formation of bicinchoninic acid-Cu¹⁺ complex at 562 nm [15].

Metabolism Assays

The enzyme reactions were conducted as previously described [11;12]. All reactions were carried out in 0.1 M Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 10 mM MgSO₄ and either a NADPH-regenerating system (1 mM β -NADPH, 0.8 mM isocitrate, and 0.1 U/mL isocitrate dehydrogenase), a NADH-regenerating system (1 mM β -NADH, 0.8 mM isocitrate, and 0.1 U/mL isocitrate dehydrogenase), or 1 mM β -NADP⁺ or β -NAD⁺. The content of each

incubation mixture was oxygenated by blowing pure O₂ into the tube for 15 seconds, the appropriate sub-cellular fraction was added, and the reaction mixture preincubated for 5 min at 37°C. Little lipid peroxidation was observed, but the enzyme assays were linear for longer times of incubation. Subsequently, various concentrations of the substrates tested (dissolved in 10 µL ethanol) were added to achieve a 1 mL volume and the incubation was continued for the desired time. Previously, with 7-hydroxy-DHEA metabolites, we found the optimal enzyme assay for rat and pig kidney and human, pig and rat livers to be at a protein concentration of 1 mg/mL for microsomes and 2 mg/mL for nuclei. In each sub-cellular fraction, the NADP⁺- and the NAD⁺-dependent oxidation of 7α-OH-DHEA to 7-oxo-DHEA and of CS to DHC, as well as, the NADPH- or NADH-dependent reduction of 7-oxo-DHEA to either 7α- or 7β-OH-DHEA and of DHC to CS was measured. The effects of 7-oxo-DHEA, CS and DHC on oxidation of 7α-OH-DHEA, and the effects of DHC, 7α-OH-DHEA and 7-oxo-DHEA on oxidation of CS were tested. Similarly, the effects of 7-oxo-DHEA 7α-OH-DHEA and CS on the reduction of DHC and the effects of 7α-OH-DHEA, CS and DHC on the reduction of 7-oxo-DHEA were assayed. For these assays, the steroid being tested as the inhibitor was added to the incubation medium (1 ml final volume) in a minimal volume of ethanol (10 µL) to attain a concentration of 50 µM. Where inhibition was observed, the inhibition kinetics were tested by providing different concentrations of the inhibiting steroid in 10 µL ethanol. The effects of the following specific inhibitors 11α-OH-PRO, 11β-OH-PRO (in 10 µL ethanol to attain a concentration of 50 µM) and CBX (2 mM dissolved in the reaction buffer) were assayed, as well [11-13;16]. The ethanol-vehicle (10 µL) also was added to the control and the CBX reaction mixtures.

The concentration dependence for added pyridine nucleotide co-substrates (β-NADP⁺ and β-NAD⁺ for dehydrogenase and β-NADPH- and β-NADH-regenerating system for reductase reactions) was assayed with those sub-cellular fractions where such reaction was found to exist. To determine the apparent *K_m* and *V_{max}* values, reactions were performed using substrate concentrations in the range of 0.2 nM - 2 µM at the optimal time determined, as determined from by monitoring the linear region of the time course for product formation. Values were determined as medians of $n(n-1)/2$ estimates of *K_m* and *V_{max}* obtained from double-reciprocal Lineweaver-Burk plots (1/*v_o* vs. 1/[S]) with 5 different [S]₀ values at a confidence level of 97% [17].

The reactions were terminated by mixing with 5 mL chilled ethyl acetate and transferring the sample to ice. For the extraction of the DHEA metabolites, the aqueous phase was then extracted three times with 5 mL ethyl acetate. For the extraction of CS and DHC, the first extraction with ethyl acetate, was followed by a second extraction with 5mL chloroform. These procedures allowed us to extract >95% of radioactivity added to incubation medium from the appropriate substrate steroid after 1-2 hours incubation with human liver protein fractions. The extracts from each metabolic assay was dehydrated by addition by addition of anhydrous Na₂SO₄ prior to concentration under a stream of nitrogen to prevent any further oxidation of the metabolites.

Thin Layer Chromatography

The dried extracts from assays of metabolism of 7-oxidized-DHEA derivatives were dissolved in 50 µL ethanol containing cold 7α-OH-DHEA, 7β-OH-DHEA and 7-oxo-DHEA (10 mM each). Dried extracts from assays of GC metabolism were dissolved in 50 µL ethanol containing cold CS and DHC (10 mM each) and the metabolites were resolved on TLC Silica gel 60 aluminum sheets (EM Science, Gibbstown, NJ, USA). The mobile phase for resolving the 7-oxidized-DHEA metabolites was chloroform:ethyl acetate:hexane:acetone:glacial acetic acid (20:5:5:5:3 V:V:V;V;V). For the separation of CS and DHC, chloroform:acetone (5:1 V:V) was used as the mobile phase. The location of each of these steroids was detected with long

wave UV light following spraying the TLC sheets with a stock solution containing 31 mg of primuline (Sigma, St. Louis, MO, USA), 120 mL water, and 3 L of acetone. The TLC media associated with the spots then was transferred into scintillation vials, scintillation fluid was added and the radioactivity was measured with a Packard Tri-CARB 2100 TR spectrometers (Dowson Groves, IL, USA). The recovery of total radioactivity from CS or 7 α -OH-DHEA added to the incubation medium that contained either human liver microsomal or nuclear protein fractions, extracted and resolved on TLC aluminum sheets, was 92-95%.

Statistical Analysis

Experiments were conducted in duplicate using samples collected from four different patients. Statistical significance was determined using ANOVA followed by student's t-test with $p \leq 0.05$ as the criterion for significance.

RESULTS

Reduction of DHC into CS by human liver microsomal protein fractions

We performed a preliminary set of experiments that demonstrated that the conversion of DHC and 7-oxo-DHEA to their respected reduced product was linear for up to an hour and at microsomal protein concentrations below 0.5-1.0 mg/mL (data not shown). Similar linear time courses were observed for the reverse reaction. This allowed us to assess the K_m and V_{max} values for several samples of human liver microsomal and nuclear protein fractions. In the presence of 1 mM reduced pyridine nucleotides, human liver microsomes catalyzed the reduction of DHC into CS at similar rates and kinetic constants with either a NADPH- or NADH-regenerating system were noted to be K_m for DHC of 7.3 ± 0.4 and 8.6 ± 0.4 μ M and V_{max} of 4.48 ± 0.12 and 3.42 ± 0.09 nmol/min/mg microsomal protein, respectively (Table 1). When this reduction reaction was assayed in the presence of varying concentrations of the pyridine nucleotide co-substrates, we observed the expected higher affinity of the enzyme for NADPH than for NADH (K_m of 142 ± 31 vs. 791 ± 182 μ M, respectively).

Oxidation of CS into DHC by human liver microsomal protein fractions

We observed that in the presence of either 1 mM NADP⁺ or NAD⁺, human liver microsomal proteins also oxidized CS to DHC at similar rates and kinetic values (K_m of 4.3 ± 0.8 and 2.8 ± 0.2 nM for CS and V_{max} of 3.25 ± 0.40 and 2.34 ± 0.08 nmol/min/mg microsomal protein, respectively (Table 1). However, when CS oxidation by human liver microsomal protein fractions was assayed with varying concentrations of the oxidized pyridine nucleotide co-substrates, the reaction showed a higher affinity for NADP⁺ than for NAD⁺ with K_m of 43 ± 8 and 264 ± 24 μ M for NADP⁺ and NAD⁺, respectively (Table 1).

Because 11 β HSD1 is co-expressed and co-localized with hexose-6-phosphate dehydrogenase in hepatocytes [18;19], its reactions are sustained by NADPH and it is assumed that it therefore acts as an intracellular reductase. However, with microsomal fractions *in vitro*, 11 β HSD1 also may act as a dehydrogenase, if only NADP⁺ co-substrate is available [2]. The rate of oxidation by human liver microsomal fractions of CS into DHC in the presence NADP⁺ was lower ($V_{max} = 3.3 \pm 0.4$ nmol product formed/min/mg protein) than was the rate of reduction of DHC into CS ($V_{max} = 4.5 \pm 0.1$ nmol CS formed/min/mg protein) when an NADPH-regenerating system was provided (Figure 1A). Similar results were observed with the metabolism of 7-oxo-DHEA, since human liver microsomal protein reduced 7-oxo-DHEA with NADPH at a rate higher than the oxidation of 7 α -OH-DHEA by NADP⁺ (Figure 1B). These results suggest that human 11 β HSD1 present in membranes of fresh human liver microsomal protein fractions and an NADPH-regenerating system serves primary as a reductase, rather than a dehydrogenase for both GC and 7-OH-DHEA as had been suggested previously for GC in a cell-free systems [2]. The characteristics of the reactions catalyzed by human liver microsomes

were concordant with this activity being due to 11 β -HSD Type 1 as we demonstrated for rat liver [9;10;12].

Glucocorticoid metabolism by human liver nuclear protein fractions

No change in DHC utilization or CS production could be detected when DHC was incubated as a sterol substrate at various concentrations (5 nM-50 μ M) for up to 4 hrs with the human liver nuclear fractions with either NADPH- or NADH-regenerating system (data not shown, Table 2). However, in the presence of 1mM NADP⁺, human liver nuclear fractions oxidized CS into DHC at a higher rate than with 1 mM NAD⁺ (Figure 2) with a K_m of 102 ± 16 and 209 ± 45 nM for CS and V_{max} of 172 ± 42 and 83 ± 8 pmol/min/mg nuclear protein, respectively (Table 2). When human liver nuclear fractions were incubated with 80 nM CS and varying concentrations of oxidized pyridine nucleotide co-substrates, the reaction showed a higher affinity for NADP⁺ (K_m of 12 ± 2 μ M) than for NAD⁺ (K_m of 390 ± 43 μ M).

The metabolism of 7-oxidized-DHEA derivatives by human liver microsomal and nuclear protein fractions

In addition to glucocorticoids, 11 β -HSD1 has been shown to metabolize 7-hydroxy/oxo-cholesterol and DHEA, accounting for their biological interconversion in rodents and humans [11;12]. In the presence of 1 mM NADPH and an NADPH-regeneration system, human liver microsomal protein fractions reduced 7-oxo-DHEA mainly to 7 β -OH-DHEA and lower amounts of 7 α -OH-DHEA (Figure 3). The K_m for the 7-oxo-DHEA consumption was 27.4 ± 4.4 μ M with a V_{max} of 4.15 ± 0.50 nmol product/min/mg protein (Table 2). Human liver nuclear protein fractions did not reduce 7-oxo-DHEA at any concentration tested when incubated in the presence of either NADPH- or NADH-regenerating system (data not shown). Both human liver microsomal and nuclear protein fractions oxidized 7 α -OH-DHEA to 7-oxo-DHEA in presence of 1 mM of NADP⁺. The rate of this reaction was higher in the human liver microsomal protein ($K_m = 20.5 \pm 2.1$ μ M; V_{max} of 1.56 ± 0.27 nmol product formed/min/mg protein) than with the nuclear protein ($K_m = 4.5 \pm 0.6$ μ M; $V_{max} = 0.17 \pm 0.03$ nmol/min/mg protein; Table 2).

Use of Inhibitors to distinguish between 11 β -HSD1 and the NADP⁺-dependent nuclear enzyme activity—There are three well characterized inhibitors thought to be specific for the 11 α -HSD family of enzymes, namely, CBX, 11 α - and 11 β -OH-PRO. In addition, other 7-oxidized sterols, such as 7-oxo-hydroxy-DHEA and 7-oxo-hydroxy-cholesterol have been suggested be competitive alternative substrates for these reactions [11; 12]. During characterization of the microsomal 11 β -HSD1 enzyme, we observed that the reduction reaction of DHC to CS was inhibited potently by carbenoxolone (CBX), 11 α -OH-PRO and 7-oxo-DHEA (Figure 4A). On the other hand, inclusion of 11 β -OH-PRO, DHC or 7 α -OH-DHEA actually augmented the yields of this reaction product, perhaps by inhibiting oxidation of its product (CS) back to DHC by competing enzymes. In presence of 1 mM NADP⁺, the oxidation of 80 nM CS was potently inhibited by CBX, 11 α - and 11 β -OH-PRO and significantly inhibited also by 7 α -OH-DHEA (Figure 4B). The suppression of DHC conversion into CS by 7-oxo-DHEA appeared to be competitive with an apparent inhibition constant, IC_{50} , of 58 ± 15 μ M (data not shown, Table 1).

These inhibitors were also used in reactions catalyzed by human liver nuclear protein fractions to establish how they affect oxidation of CS and 7 α -OH-DHEA by NADP⁺. The reduction of 7-oxo-DHEA into 7 α - and 7 β -OH-DHEA was partially inhibited by CBX, potently inhibited by 11 α -OH-PRO and DHC, but not by 11 β -OH-PRO (Figure 5). The reaction was enhanced by inclusion of CS and 7 α -OH-DHEA, perhaps by competitive inhibition of the reverse oxidation. The suppression of the oxidation of CS by 7 α -OH-DHEA was competitive with an apparent inhibition constant, IC_{50} , of 81 ± 29 μ M for -OH-DHEA 7 α (Table 1, data not shown).

This competitive inhibition also may explain the increased yields of 7 α -OH-DHEA, but not of 7 β -OH-DHEA, with the addition of unlabelled 7 α -OH-DHEA (Figure 5). Human liver nuclear protein fractions did not reduce 7-oxo-DHEA at any concentration tested when incubated in the presence of either NADPH- or NADH-regenerating system (data not shown).

The characteristics of the nuclear enzyme activity toward CS oxidation was also noted to be significantly affected by these same inhibitors, CBX, 11 α - and 11 β -OH-PRO (Figure 6A.) 7 α -OH-DHEA also caused a 50-55% inhibition of CS oxidation similar to the results seen with the microsomal enzyme. The oxidation of 7 α -OH-DHEA into 7-oxo-DHEA by human liver nuclear protein was also abolished completely by 2 μ M CBX and by 50 μ M of either 11 α -OH-PRO, 11 β -OH-PRO or DHC. However, the reaction was inhibited partially by 50 μ M DHC or 7-oxo-DHEA (Figure 6B). The oxidation of 7 α -OH-DHEA into 7-oxo-DHEA by human liver microsomes was abolished completely by 2 μ M CBX, 50 μ M 11 α -OH-PRO or 11 β -OH-PRO, or 50 μ M CS (Figure 6C). The reaction yields were enhanced by inclusion of 50 μ M DHC or 7-oxo-DHEA into the reaction mixture, most likely due to competition with reduction of radiolabeled 7-oxo-DHEA formed by the reverse reduction with human liver microsomal protein fractions. The enzyme activity in human liver nuclei appears to be due to the presence of a unique HSD enzyme activity, possibly 11 β -HSD Type 3, since it does not catalyze reduction of DHC or 7-oxo-DHEA like the Type 1 enzyme even in the presence of an NADPH-regenerating system and has a preference for β -NADP⁺ over β -NAD⁺ unlike the Type 2 enzyme. Its sensitivity to the inhibitors of 11 β -HSD was similar to that observed with microsomal 11 β -HSD1, suggesting that it may be a variant of 11 β -HSD1 or a gene whose critical substrate binding sites are similar to 11 β -HSD.

DISCUSSION

Previously, we demonstrated that 7 α -hydroxy-cholesterol is a substrate for purified hamster liver 11 β HSD1 [20]. We subsequently observed that human liver microsomes serve as oxidoreductases with 7-oxidized-DHEA metabolites [9-12]. When provided with an NADPH-regenerating system, human liver microsomes converted 7-oxo-DHEA principally to 7 β -OH-DHEA, and at a lesser extent to 7 α -OH-DHEA. When NADP⁺ was used as an oxidizing cofactor, human liver microsomal protein oxidized 7 α - and 7 β -OH-DHEA into 7-oxo-DHEA. The K_m for 7-oxo-DHEA in its reduction to 7-hydroxy-DHEA was 27.4 ± 4.4 μ M, three to four times higher than the K_m for DHC in its reduction to CS (7.3 ± 0.4 μ M), although the rates of oxidation (V_{max}) for both reactions were similar (4.48 ± 0.12 and 4.15 ± 0.50 nmol sterol formed/min/mg protein for DHC and 7-oxo-DHEA, respectively). In the presence of NADP⁺, the K_m for 7 α -OH-DHEA in its oxidation into 7-oxo-DHEA also was higher than that observed for CS in the oxidation of CS into DHC (20.5 ± 2.1 vs. 4.2 ± 0.8 μ M) and the V_{max} for the CS oxidation (3.25 ± 0.40 pmol product formed/min/mg protein) was twice that observed for oxidation of 7 α -OH-DHEA into 7-oxo-DHEA (1.56 ± 0.27 pmol product formed/ml/mg protein). Therefore, the efficiency of catalytic processing of 7-oxidized-DHEA metabolites are lower than for GC. This suggests that the primary action of 11 β HSD1 in the human liver is to convert GC from their inactive 11-keto-derivative into the active 11-hydroxy derivative. However, the metabolism of 7-oxidized-DHEA metabolites by human liver microsomal proteins may well be another normal function of the enzyme, but its physiological significance requires further study. With NADPH, human liver microsomal protein reduced 7-oxo-DHEA at a rate higher than the oxidation of 7 α -OH-DHEA by NADP⁺.

Walker *et al.* [21] successfully expressed human 11 β -HSD Type 1 in *E. Coli* and after purification, characterized the kinetic properties of the purified enzyme. The K_m for cortisol was observed to be 1.4 μ M and for corticosterone was 9.5 μ M; values similar to those we observed of 7.3 μ M for DHC reduction by human liver microsomal fractions in the range reported previously for human and hamster liver microsomal protein fractions metabolizing

cortisone and cortisol (2.5-9.5 μM) [20;22]. Walker *et al.* [21] also demonstrated that the purified enzyme, while stated to be somewhat unstable, it could catalyze both the reduction of corticosterone and oxidation of cortisol. These results clearly demonstrate that in the presence of NADPH, 11 β HSD Type I displays the ability membranes and purified state. In contrast, the activity of the nuclear enzyme activity we observed did not display the reduction reaction in the presence of NADPH, but does utilize NADP⁺ as an oxidizing substrate. Our experiments were performed in the presence of NADPH and an NADPH-regenerating system that should have provided sufficient reducing equivalents to HSD activity. The nuclear enzyme did not catalyze reduction of DHC with NADPH, but did oxidize CS with NADP⁺.

We also observed an NAD⁺-dependent, unidirectional 11 β HSD that oxidizes CS into DHC and 7 α -OH-DHEA into 7-oxo-DHEA in the nuclear fraction of rat and pig kidney; however, these fractions do not catalyze the reduction of DHC or 7-oxo-DHEA [11;12], suggesting that this activity may be due to 11 β HSD2, an enzyme thought to prevent GC activation of kidney MR and oxidize 7-hydroxy-DHEA [1;23]. In the current study, we found a second unidirectional 11 β HSD activity in human liver nuclear protein fractions, suggesting that the nucleus of human hepatocyte is equipped with a 11 β HSD that oxidizes and deactivates GC. This nuclear 11 β HSD is of high-affinity and low-capacity (K_m of 102 ± 16 nM and V_{max} of 172 ± 42 pmol product produced/min/mg protein), similar to the rate previously reported for 11 β HSD2 and 3 in kidney [11;12;24]. Because this enzyme has a much higher affinity for NADP⁺ than for NAD⁺ (K_m for the co-substrate of 12 ± 2 μM vs. 390 ± 43 μM , respectively), it appears to be similar to the putative 11 β HSD3 observed in rat, pig and sheep kidney [7;11;12]. Therefore, the presence of an enzyme activity distinctly different from 11 β -HSD Type 1 or 2 in the nuclear membrane of the human hepatocyte may prevent *in situ* activated GC from penetrating the nucleus and altering gene expression.

Because CBX [25] drastically diminished the reduction and oxidation of GC and 7-oxidized-DHEA metabolites by human liver microsomes and the oxidation of CS and 7 α -OH-DHEA by human liver nuclear protein, we assume that this nuclear enzyme activity is due to a member of the 11 β HSDs family, since all 3 enzymes displayed similar responses to known 11 β -HSD inhibitors. 11 α - and 11 β -OH-PRO were also shown to be potent specific inhibitors of 11 β HSDs [16]. Previously, we observed that at a concentration of 50 μM , both 11 α - and 11 β -OH-PRO almost totally abolished the conversion of CS into DHC and of 7 α -OH-DHEA to 7-oxo-DHEA by pig kidney nuclei and microsomes [11]. In the human liver, we found the 11 α - and 11 β -OH-PRO did not display as high levels of inhibition on metabolism of GC and 7-oxidized-DHEA by 11 β HSDs. Both 11 β - and 11 β -OH-PRO inhibited CS oxidation into DHC and totally abolished 7 α -OH-DHEA oxidation into 7-oxo-DHEA by human microsomes and nuclear protein fractions. However, only 11 α -OH-PRO inhibited the reduction of DHC to CS and of 7-oxo-DHEA into 7 β - and 7 α -OH-DHEA by human liver microsomes. In contrast, 11 β -OH-PRO had no significant effect of the reduction of the 7-oxo-DHEA and even augmented the yield of CS during DHC reduction into CS, possibly by inhibiting the reverse oxidation. Therefore, 11 α -OH-PRO apparently inhibits both the dehydrogenase and the reductase action of human hepatic 11 β HSDs, while 11 β -OH-PRO derivative probably [11;12]inhibits the oxidation of CS and 7-OH-DHEA by 11 β HSD1, 2 and 3, but not the reduction of DHC and 7-oxo-DHEA by human 11 β HSD1.

As we previously reported [11;12], GC compete with the 7-oxidized metabolites of DHEA during the catalytic action of these dehydrogenases and *vice versa*. In the current study, we documented for the first time that 7-oxidized-DHEA metabolites competitively inhibit GC processing by the human liver 11 β HSDs. In a similar manner, 7 α -OH-DHEA competitively inhibited the oxidation, and therefore, the deactivation of GC at both the microsomes and the nuclei of the human liver. Recently, Seckl and coworkers demonstrated that 7-oxo-cholesterol can serve as a competitive inhibitor of 11 β HSD1 in human adipocytes [26]. This suggests that

in addition to cortisone and cortisol, 7 α -hydroxy-cholesterol, 7 α -hydroxy-DHEA, and structurally related sterols may be substrates for the 11 β HSDs, and under pathophysiological conditions may alter each other's metabolism.

Hepatic 11 β HSD1 has a much broader role than just the metabolism of GC, 7-oxidized-DHEA and 7-oxidized-cholesterol. Its reductase function is involved in the metabolism and/or detoxification and excretion of xenobiotics, drugs, insecticides and carcinogens (for review see [2]. Since 11-keto-GC and 7-oxo-DHEA inhibit this function in human liver microsomes, consumption of pharmacological amounts of either 7-keto-DHEA or 11-keto-GC, such as prednisone, may render humans more susceptible to the toxic effects of pollutants and even some drugs [11;27]. Therefore, one may assume that when individuals are prescribed 11-keto-GC derivatives, physicians should consider any contraindication of 7-oxidized-DHEA metabolite taken by the patient as a nutraceutical, since these agents may ameliorate the conversion of the inactive 11-keto-GC to its reduced active form (e.g. the conversion of prednisone into prednisolone) as well as the clearance of xenobiotics, drugs, insecticides and carcinogens.

7-Ketocholesterol accumulation is involved in several pathophysiological events and is found in human atherosclerotic plaques [28]. It was suggested that its conversion into 7 β -OH-cholesterol by 11 β HSD1 initiates its conversion to more polar, water soluble products for elimination via the bile acid pathway [29;30]. Inhibition of 11 β HSD1 has been suggested as a possible cause for accumulation of 7-ketocholesterol in human subjects [18;20;31]. The competitive inhibition of 11 β HSD1 reductase activity by 11-keto-GC or other exogenously administered compounds may also result in elevated 7-ketocholesterol levels and might enhance development of the atherosclerosis as seen following long-term administration of prednisone [32]. Finally, 11 β HSD1 in adipocytes seems to be involved in the development of obesity and the general metabolic syndrome [33], and inhibition of 11 β HSDs in the adipose tissue may provide new methods for treating such conditions [34;35]. For therapeutic use, inhibitors have to be designed to be tissue and/or organelle specific [35], otherwise they may inhibit 11 β HSD1 in other tissues, including the liver, resulting in deficiency in 11-OH-GC, reduction of 7 β -OH-DHEA levels, inhibition in detoxification of xenobiotics, drugs, insecticides and carcinogens, and accumulation of 7-ketocholesterol.

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Abbreviations

CS, Corticosterone
 DHC, dehydrocorticosterone
 CBX, carbenoxolone
 DHEA, dehydroepiandrosterone
 7-OH-DHEA, 7-hydroxy-DHEA
 7 α -OH-DHEA, 7 α -hydroxy-DHEA
 7 β -OH-DHEA, 7 β -hydroxy-DHEA
 GC, glucocorticoids
 11 β HSD, 11 β -hydroxysteroid dehydrogenase
 11 α -OH-PRO, 11 α -hydroxyprogesterone
 11 β -OH-PRO, 11 β -hydroxyprogesterone
 ER, endoplasmatic reticulum

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Figure 1.

Concentration dependence of oxidative and reductive metabolism of 11 β -HSD substrates by human liver microsomal fractions. Human liver protein (0.5 mg/mL) was incubated for 120 min with either 1 mM β -NADPH min regenerating system or 1 mM β -NADP⁺ as described in the method section. A. Oxidation of CS by β -NADP⁺ [Δ] or reduction by β of DHC by β -NADPH [$\circ$]. B. Oxidation of 7 α -OH-DHEA by β -NADP⁺ [δ] or reduction of 7-oxo-DHEA by β -NADPH [$\circ$]. The rate of metabolism was expressed nmol product formed per mg protein per minute (mean value $\pm$ SEM; n=5).

Figure 2.

Concentration dependence of oxidation of CS to DHC by human liver nuclei. Human liver nuclei (0.5 mg/mL) were incubated with either 1 mM β -NADP⁺ or 1 mM β -NAD⁺ for 90 min as described in the method section. Product formation was expressed as pmol product formed per mg protein per min (mean value $\pm$ SEM; n=5).

Figure 3.

Concentration dependence of 7-oxo-DHEA reduction to 7 α - and 7 β -OH-DHEA by human liver microsomal fractions. Human liver microsomes (0.5 mg/mL) were incubated with 1 mM β -NADPH and a regenerating system in the presence of varying concentrations of 7-oxo-DHEA for 90 min as described in the method section. Product formation was expressed as pmol 7-oxo-DHEA utilized [▲], 7 α -OH-DHEA [■] or 7 β -OH-DHEA [●] formed per mg protein per min (mean value $\pm$ SEM; n=5).

Figure 4.

The effects of 11 β -HSD on the metabolism of corticosterone and dehydrocorticosterone by human liver microsomal fractions. Liver microsomal fractions from human liver were incubated with either 1 mM β -NADPH and 10 μ M DHC or 1mM β -NADP⁺ and 10 μ M CS for 120 min in the absence or presence of CBX (2 μ M) or 50 μ M of either 11 α -hydroxy-progesterone, 11 β -hydroxy-progesterone, dehydrocorticosterone, 7-oxo-DHEA or 7 α -OH-DHEA as described in the method section. **A.** Inhibition of the reduction of DHC by NADPH. **B.** Inhibition of the oxidation of CS by NADP⁺. The rates were expressed as nmol CS or DHC formed per mg protein per min (mean value $\pm$ SEM; n=5). Statistical significance, *, p < 0.01.

Figure 5.

Effects of inhibitors on the reductive metabolism of 7-oxo-DHEA to 7α-OH-DHEA and 7β-OH-DHEA by human liver microsomal protein in the presence of 1 mM β-NADPH. Human liver microsomal protein was incubated for 120 min with 100 μM 7-oxo-DHEA in the absence or presence of either CBX (2 μM) or 50 μM of either 11α-OH-PRO, 11β-OH-PRO, DHC, CS or 7α-OH-DHEA as described in the method section. The reaction rates were expressed as % control the rates of reduction of 7-oxo-DHEA (mean value ± SEM; n=5). Statistical significance, *, p < 0.01.

Figure 6.

The effects of inhibitors on the conversion of CS to DHC or 7 α -OH-DHEA to 7-oxo-DHEA by human liver microsomal and nuclear protein with 1 mM β -NADP⁺. Human liver microsomal or nuclear fraction protein (0.5 mg/mL) was incubated with 1 mM β -NADP and either 80 nM CS or 100 μ M 7 α -OH-DHEA for 120 minutes in the absence or presence of CBX (2 μ M) or 50 μ M of either 11 α -OH-PRO, 11 β -OH-PRO, DHC, CS or 7-oxo-DHEA as described in the method section. A. Human liver nuclear protein (ND, not determined), B. Human liver microsomal protein. The rates of sterol oxidation were expressed as nmol DHC formed per mg per min or pmol 7-oxo-DHEA formed per mg protein per minute (mean value $\pm$ SEM; n=5). Statistical significance, *, $p < 0.01$.

The kinetic constants for corticosterone/dehydrocorticosterone and 7-OH-DHEA/7-oxo-DHEA oxidation or reduction by human liver microsomal protein fractions

The values were determined as described in the materials and methods section. The results were obtained with duplicate measurements at varying substrate concentration using separate liver microsomal protein fractions from 4 individuals. The standard deviation of the values was less than 10% of the mean value.

Table 1

Reaction	Cofactor	V _{max} (nmol/min/mg)	K _m (μM) Sterol	K _m (μM) Pyridine nucleotide	K _i (μM)
Human Liver Microsomes					
DHC → CS	NADPH	4.48 ± 0.12	7.3 ± 0.4	142 ± 31	58 ± 15 7-oxo-DHEA
DHC → CS	NADH	3.42 ± 0.09	8.6 ± 0.4	791 ± 182	
CS → DHC	NADP ⁺	3.25 ± 0.40	4.3 ± 0.8	43 ± 8	81 ± 29 7α-OH DHEA
CS → DHC	NAD ⁺	2.34 ± 0.08	2.8 ± 0.2	264 ± 24	
7-oxo-DHEA → 7OH-DHEA	NADPH	4.15 ± 0.50	27.4 ± 4.4		
7-OH DHEA → 7-oxo-DHEA	NADP ⁺	1.56 ± 0.27	20.5 ± 2.1		

Table 2**The kinetic constants for corticosterone and 7-OH-DHEA oxidation by human liver nuclear protein fractions**

The values were determined as described in the materials and methods section. The results were obtained with duplicate measurements at varying substrate concentration using separate liver nuclear protein fractions from 4 individuals. The standard deviation of the values was less than 10% of the mean value.

Reaction	Cofactor	$V_{\max}$ (nmol/min/mg)	K_m (μ M) Sterol	K_m (μ M) Pyridine nucleotide
Human Liver Nuclear protein				
DHC $\rightarrow$ CS	NAD(P)H	No Reaction		
CS $\rightarrow$ DHC	NADP ⁺	0.17 ± 0.04	0.10 ± 0.02	12 ± 2
CS $\rightarrow$ DHC	NAD ⁺	0.083 ± 0.08	0.21 ± 0.05	390 ± 43
7-oxo-DHEA $\rightarrow$ 7OH-DHEA	NADPH	No Reaction		
7-OH DHEA $\rightarrow$ 7-oxo-DHEA	NADP ⁺	0.17 ± 0.03	4.5 ± 0.6	