Published in final edited form as:

Arch Biochem Biophys. 2005 October 1; 442(1): 33–40.

Interactions between dehydroepiandrosterone and glucocorticoid metabolism in pig kidney: Nuclear and microsomal 11 β -hydroxysteroid dehydrogenases

Boaz Robinson¹ and Russell A. Prough²

¹Department of Animal Science, Faculty of Agricultural, Food and Environmental Quality Sciences, The Hebrew University of Jerusalem, P.O.B. 12, Rehovot 76100, Israel, Phone: 972-8-9489396; Fax 972-8-9465763; Email: robinzon@agri.huji.ac.il

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

Abstract

The 11 β -hydroxysteroid dehydrogenase type 1 (11 β HSD1) activates glucocorticoids (GC) by reversibly converting 11-keto-GC to 11-hydroxy-GC, while 11 β HSD2 and 11 β HSD3 only catalyzes the reverse reaction. Recently, rat and human 11 β HSDs were shown to interconvert 7 α - and 7 β -hydroxy-dehydroepiandrosterone (7 α - or 7 β -OH-DHEA) with 7-oxo-DHEA. We report that pig kidney microsomes (PKMc) and nuclei (PKN) oxidize 7 α -OH-DHEA to 7-oxo-DHEA at higher rates with NAD⁺, than with NADP⁺. Corticosterone (CS), dehydrocorticosterone (DHC), 11 α - and 11 β -hydroxyprogesterone, and carbenoxolone completely inhibited these reactions, while 7-oxo-DHEA only inhibited the NAD⁺-dependent reaction. Conversely, CS oxidation was not inhibited by 7 α -OH-DHEA or 7-oxo-DHEA. PKMc and PKN did not convert 7-oxo-DHEA to 7-OH-DHEA with either NADPH or NADH. Finally, PKN contained a high affinity, NADPH-dependent 11 β HSD that reduces DHC to CS. The GC effects on interconversion of DHEA metabolites may have clinical significance, since DHEA and its 7-oxidized derivatives have been proposed for treatment of human autoimmune and inflammatory disorders.

Keywords

Corticosterone; dehydrocorticosterone; dehydroepiandrosterone; DHEA; 7 α -hydroxy-DHEA; 7 β -hydroxy-DHEA; 7-oxo-DHEA; 11 β -hydroxysteroid dehydrogenase; kidney; pig

INTRODUCTION

Multiple isozymes of the 11 β -hydroxysteroid dehydrogenases (11 β HSDs) have been described that regulate the metabolism of GC. The 11 β HSDs are membrane-bound, pyridine nucleotide-dependent dehydrogenase enzymes found in endoplasmic reticulum and other membrane organelles [1,2]. In liver, the principle isozyme observed is the microsomal 11 β HSD1 that catalyzes activation or deactivation of GC, depending on the presence of either NADP⁺ or NADPH. It has been suggested that microsomal 11 β HSD1 acts as a reductase, when coupled

Cite this article as: B. Robinson, R.A. Prough, Interactions between dehydroepiandrosterone and glucocorticoid metabolism in pig kidney: Nuclear and microsomal 11 β -hydroxysteroid dehydrogenases, *Archives of Biochemistry and Biophysics*, 10.1016/j.abb.2005.07.010

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review before it published in its final citable form. Please note that during the production process errors may be discovered which could affect the content and all legal disclaimers that apply to the journal pertain.

in microsomal vesicles with hexose-6-phosphate dehydrogenase activity as a source of NADPH [3,4]. In kidney, 11 β HSD1 serves as an NADP⁺-dependent dehydrogenase, since it is apparently not coupled with hexose-6-phosphate dehydrogenase.

Other isozymes of 11 β HSDs catalyze unidirectional reactions that are solely dependent upon NAD⁺ as oxidizing cofactors. The major role proposed for 11 β HSD2 and 3 is to convert GC from their active alcohol form (cortisol and corticosterone) to the keto non-active form (cortisone and dehydrocorticosterone) in an irreversible reaction, thereby, preventing binding of GC to the mineralocorticoid receptor. 11 β HSD activity has been observed in nuclei, mitochondrial and microsomal organelles from normal human and rat placenta [5] and choriocarcinoma cells [6]. In human kidney nuclei, immuno-reactive human 11 β HSD2 accounted for 40% of total cellular 11 β HSD2 protein content [7,8]. In kidney from rat and sheep, a third dehydrogenase (11 β HSD3) was shown to be present in membranes of Golgi apparatus and mitochondria, while 11 β HSD2 is preferentially located in the nuclear membrane [9,10]. Because of their differing sub-cellular distribution and function in GC metabolism, 11 β HSDs are considered gatekeepers that prevent excess GC in plasma from binding to nuclear MR. However, the NADPH-dependent 11 β HSD1 activity allows formation of GC, thereby serving as a pre-receptor regulated process.

Recently [9,11], we demonstrated conversion of DHEA to 7 α -OH-DHEA in human, pig and rat liver microsomal fractions, and to a lesser extent, in rat liver mitochondria (RLMw) and nuclei (RLN). 7 α -Hydroxy-DHEA can then be further converted to 7-oxo-DHEA by 11 β HSD1 in rat liver microsomes (RLMc), by 11 β HSD2 in rat kidney nuclei (RKN) and by 11 β HSD3 in rat kidney mitochondria (RKMw) and microsomal fractions (RKM_c). In liver microsomes, 7-oxo-DHEA was reduced by 11 β HSD1 and other oxidoreductases in a species-specific manner [9]. PLMc reduced 7-oxo-DHEA to nearly equal amounts of 7 α - and 7 β -OH-DHEA, while HLMc reduced it mainly to 7 β -OH-DHEA. In contrast, RLMc rapidly convert 7-oxo-DHEA in the presence of NADPH to several, yet unidentified, oxidized metabolites, perhaps by cytochromes P450. Since 11 β HSDs are involved, the reaction was inhibited by the specific inhibitor of 11 β -HSDs, carbenoxolone (CBX). The GC, probably by serving as substrates for 11 β HSDs, also inhibited the rate of metabolism of DHEA-derivatives by all fractions from all examined species, in what appeared to be a competitive process [9].

Since the human kidney is a target tissue for inflammation and autoimmune disorders [12], the metabolism of DHEA and its 7-oxidized metabolites in kidney was studied. In this report, we present data on the interconversion of 7-hydroxy- and 7-oxo-DHEA showing that the pig kidney contains several different membrane-bound dehydrogenases with enzyme activities similar to other members of the 11 β HSD family. Specific 11 β HSD inhibitors, as well as DHC, completely block oxidation of 7 α -OH-DHEA, while causing partial inhibition of CS conversion to DHC. Finally, these results demonstrate that porcine kidney nuclei also contain a high affinity, low capacity NADPH-dependent 11 β HSD that reduces DHC to CS.

MATERIALS AND METHODS

Chemicals: DHEA, 7 α -OH-DHEA, 7 β -OH-DHEA and 7-oxo-DHEA, 11 α -hydroxy-progesterone (11 β -OH-PRO), 11 β -hydroxy-progesterone (11 β -OH-PRO), CS and DHC were obtained from Steraloids Inc. (Wilton, NH). Carbenoxolone (CBX), β -NADPH, β -NADP⁺, β -NAD⁺ and β -NADH were purchased from Sigma Chemical Co., Inc., (St. Louis, MO). HPLC grade hexane, ethyl acetate, ethanol, chloroform and acetone were obtained from Fisher Scientific Co. (Pittsburgh, PA). [1,2,6,7-³H]-DHEA and [1,2,6,7-³H]-CS were purchased from NEN Life Science Products (Boston, MA). Tritiated 7 α -OH-DHEA and 7-oxo-DHEA were prepared by the method developed by our laboratory [9]. In brief, [1,2,6,7-³H]-DHEA was incubated with PLMc with an NADPH-regenerating system (1 mM β -NADPH, 0.8 mM

isocitrate, and 0.1 U/mL isocitrate dehydrogenase) and 1mM NADP⁺ for production of both 7 α -OH-DHEA and 7-oxo-DHEA. The metabolites were extracted with three volumes (5 ml each) of ethyl acetate and then 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 comigrated 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 that contained the expected 7-oxidized metabolite as the only detectable sterol. 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 PLMc in 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 were then 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 a specific radioactivity of 5.63 mCi/mmol.

Tissues: Liver and kidney tissues from pigs were collected at a local slaughterhouse (KY Bison Co, Memphis, IN) and after the excess connective tissue was dissected away, the kidney was immediately cut into small pieces and flash frozen in liquid nitrogen, prior to storage at -70° C .

Preparation of subcellular fractions: The tissues were processed as described previously [9]. The tissues 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 $1,000 \times g$ for 10 min and after the supernatant was removed, the nuclear pellet was 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 to obtain the mitochondrial pellet, and the remaining supernatant was centrifuged at $18,000 \times g$ for another 20 min. The resulting supernatant then was sedimented at $108,000 \times g$ for 60 min to obtain the microsomal pellet and cytosol fraction. Each mitochondrial and microsomal pellet was resuspended in the wash buffer, and sedimented a second time under the same conditions. After resuspension of each pellet in preservation buffer at the volume equal to the original tissue weight, the samples were stored in aliquots at -70° C prior to assay. All fractions were stored at -70° C. Protein concentrations was determined by measuring formation of bicinchoninic acid-Cu¹⁺ complex at 562 nm [13]. The presence of microsomal and mitochondrial protein cross-contamination was assayed by testing NADH- and NADPH-dependent cytochrome *c* reductase [14] and succinate-cytochrome *c* reductase [15] enzyme activities, accordingly.

Metabolism Assays: The enzyme reactions were conducted as previously described [9]. All reactions were carried out in 0.1 M Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 10 mM MgSO₄ and either NADPH-regenerating system (1 mM β -NADPH, 0.8 mM isocitrate, and 0.1 U/mL isocitrate dehydrogenase), NADH-regenerating system (1 mM β -NADH, 0.8 mM isocitrate, and 0.1 U/mL isocitrate dehydrogenase), or either 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. Then, various concentrations of the tested substrate (dissolved in 10 μ L ethanol) were added to achieve a 2 mL volume and the incubation was continued for the desired time. Previously, with 7-hydroxy-DHEA metabolites, we found optimal enzyme activity for

rat kidney and human, pig and rat livers, when the protein concentration was 1 mg/mL for microsomes and 2 mg/mL for mitochondria and nuclei fractions. In each sub-cellular fraction, the NADP⁺- and the NAD⁺-dependent oxidation of 7 α -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 7 α - or 7 β -OH-DHEA and of DHC to CS was measured. The effects of 7-oxo-DHEA, CS and DHC on oxidation of 7 α -OH-DHEA and the effects of DHC, 7 α -OH-DHEA and 7-oxo-DHEA on oxidation of CS were tested.

For these assays, the steroid being tested as an inhibitor was added to the incubation medium (2 ml final volume) in a minimal volume of ethanol (10 μ L) to attain a concentration of 50 μ M (11 α -OH-PRO, 11 β -OH-PRO, 7 α -hydroxy-DHEA, 7-oxo-DHEA or CS). The control reaction mixtures had the vehicle alone added. For experiments using CBX as an inhibitor, CBX (2 mM) was dissolved in the reaction buffer [7,9]. The effect of adding both pyridine nucleotide co-substrates (1 mM β -NADP⁺ plus 1mM β -NAD⁺) to an incubation mixture was compared to reaction mixtures used to measure CS and 7 α -OH-DHEA oxidation with pig kidney microsomes (PKMc) and nuclei (PKN).

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, following the first extraction with ethyl acetate, a second extraction with 5 mL chloroform was made. These procedures allowed us to extract >95% of radioactivity added to incubation medium from the appropriate substrate steroid after 2 hours incubation with PLMc or PKMc. The extracts from each metabolic assay was dried of water with 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 μ L ethanol containing cold 7 α -OH-DHEA, 7 β -OH-DHEA and 7-oxo-DHEA (10 mM each) to achieve a final volume of 50 μ L. Dried extracts from assays of GC metabolism were dissolved in 50 μ L 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). The mobile phase for resolving the 7-oxidized-DHEA metabolites was ethyl acetate:hexane:glacial acetic acid 18:8:3 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), 120 mL water, and 3 L of acetone. The TLC media associated with the spots were then transferred into scintillation vials, scintillation fluid was added and the radioactivity was measured with a Packard Tri-CARB 2100 TR spectrometers (Dowson Groves, IL). The recovery of radioactive CS or 7 α -OH-DHEA added to medium with microsomal fraction extracted and resolved on TLC aluminum sheets was >95% of that expected.

Statistical Analysis. Experiments were conducted in duplicates using samples collected from 3-6 different animals. Statistical significance was determined using ANOVA followed by student's t-test with $p \leq 0.05$ as the criterion for significance.

RESULTS

In order to document the purity of the various membrane preparations, we used two enzyme assays to establish the amount of potential cross-contamination of kidney nuclei, mitochondria (succinate:cytochrome *c* oxidoreductase activity) and microsomal fractions (NADPH:cytochrome *c* oxidoreductase activity) for this study. The kidney nuclear membrane fraction contained little or no microsomal or mitochondrial cytochrome *c* oxidoreductase activity based on these assays (Table 1). In the kidney microsomal fraction, appreciable

amounts of mitochondrial enzyme activity was evident, since the level of succinate:cytochrome *c* oxidoreductase activity was approximately 26% of that seen in the mitochondrial fraction. In kidney mitochondrial fractions, NADPH:cytochrome *c* oxidoreductase enzymatic activity was noted to be about 38% of that seen for microsomal fractions. Similar amounts of cross contamination of microsomal and mitochondrial enzyme activities have been observed by others in these organelle fraction [14,15]. Although there was some cross-activity from the membrane fractions observed between microsomal and mitochondrial fractions, the nuclei fractions displayed little cross-contamination by microsomal and mitochondrial fractions.

As seen in Fig 1, PKMc converted 7 α -OH-DHEA (50 μ M initial concentration in a 2 ml reaction mixture) to 7-oxo-DHEA at higher efficiency when incubated for up to 4 hours with NAD⁺ (29.1 ± 3.1 nmol/mg protein), than when incubated with NADP⁺ (7.2 ± 1.4 nmol/mg protein). Similarly, the conversion by PKN was 22.5 ± 0.5 nmol/mg protein with NAD⁺ versus 4.9 ± 0.7 nmol/mg protein with NADP⁺. In contrast, the conversion rate by the mitochondrial fraction (PKMw) was much lower; 3.5 ± 0.7 nmol/mg protein and 1.4 ± 0.1 nmol/mg protein in the presence of either NAD⁺ or NADP⁺.

Addition of CS (50 μ M) to the medium prior to incubation nearly completely inhibited the conversion of 7 α -OH-DHEA to 7-oxo-DHEA in all sub-cellular fractions incubated with either NAD⁺ or NADP⁺ (Figure 2). When we assayed conversion of CS to DHC by PKMc in presence of NAD⁺, CS was added to achieve a concentration of 50 μ M in the incubation medium. Under this condition, 1.3 ± 0.1 nmol/mg protein was converted to DHC within the first 40 min and no further conversion was observed for up to 240 min of incubation (data not shown). However, when the assay was performed with CS at a substrate concentration of 2 μ M (specific radioactivity of 5.6 mCi/mmol), a linear conversion of CS to DHC was observed up to 60 min. (Fig 3) and total conversion for 240 min was 2.86 ± 31 nmol/mg protein. This conversion was more than double that observed when CS substrate concentration was 25 times higher, suggesting inhibition by the substrate (CS) or its product (DHC). These results suggest that the conversion of 7 α -OH-DHEA to 7-oxo-DHEA is catalyzed by low-affinity/high capacity enzyme(s), while the oxidation of CS to DHC may involve high-affinity/low capacity enzyme (s) as has been described for 11 β HSD2 [29].

The effect of addition of either DHC, 7 α -OH-DHEA or 7-oxo-DHEA (final concentration 50 μ M) prior to the administration of CS (2 μ M) as substrate was determined, as seen for PKMc (Figure 4A) and with PKN (Figure 4B) with either NAD⁺ or NADP⁺ for a 120 min reaction. In controls, conversion rate of CS to DHC was similar in both fractions and with either of the pyridine nucleotide co-substrates. In both fractions with either NAD⁺ or NADP⁺, prior administration of DHC dramatically inhibited the conversion rate. In contrast, 7 α -OH-DHEA or 7-oxo-DHEA had little or no significant effect on the conversion of CS to DHC.

The effects of 50 μ M DHC or 7-oxo-DHEA on oxidation of 7 α -OH-DHEA by PKMc or by PKN, with either NAD⁺ or NADP⁺, are presented in Figure 5. As observed before, in controls of both fractions, the conversion rate was much higher with NAD⁺ as co-substrate than with NADP⁺. Under all conditions, DHC almost totally inhibited the reactions. On the other hand, with either fractions, 7-oxo-DHEA caused about 50% inhibition of 7 α -OH-DHEA oxidation when NAD⁺ was the co-substrate, but had much less effect on the NADP⁺-dependent reaction.

No reduction of 7-oxo-DHEA to either 7 α -OH-DHEA or 7 β -OH-DHEA and no loss in the amount of added 7-oxo-DHEA could be detected during 240 min of incubation with either PKMc or PKN in presence either NADPH- or NADH-regenerating systems (data not shown). Very low, but significant rates of reduction of DHC to CS (84 ± 18 pmol/mg protein/120 min) were noted for the nuclear fraction in presence of NADPH. However, no reduction of DHC

could be detected when it was incubated with PKN in presence of NADH or with PKMc in presence of either NADPH or NADH.

As the results suggested that the oxidation of 7 α -OH-DHEA to 7-oxo-DHEA and of CS to DHC are catalyzed by two different enzymes, the effects of specific 11 β HSDs inhibitors, carboxolone (CBX), 17 α -progesterone (17 α -OH-PRO), and 17 β -progesterone (17 β -OH-PRO), on both reactions catalyzed by PKN and PKMc were tested. The conversion of 7 α -OH-DHEA to 7-oxo-DHEA was totally inhibited by all three specific 11 β HSDs inhibitors used (100% inhibition by 2 μ M CS or 50 μ M of either 11 α - or 11 β -OH-PRO), with either NAD⁺ or NADP⁺ as co-substrate (data not shown). These specific inhibitors also potently diminished the conversion of CS to DHC (Table 2). Both 11 α - and 11 β -OH-PRO completely abolished the conversion of C to DHC by PKN in presence of either NAD⁺ or NADP⁺, but the only partially inhibited the reaction catalyzed by PKMc. This was even more obvious for the CBX that totally inhibited oxidation of 7 α -OH-DHEA and CS in the PKN, but only partially inhibited conversion of CS to DHC in the PKMc, where 89% inhibition was noted in the presence of NAD⁺ and 86% inhibition in presence of NADP⁺.

As observed earlier, the reaction rate for conversion of 7 α -OH-DHEA to 7-oxo-DHEA was much higher when NAD⁺ served as cosubstrate, than in the presence of NADP⁺ (Table 3). Addition of both pyridine nucleotide co-substrates together, did not increase rate of conversion of either metabolite (7 α -OH-DHEA or CS) in either PKN or PKMc, beyond the conversion rate observed with NAD⁺ alone (Table 3). Without pyridine nucleotide co-substrate, no significant oxidation of 7 α -OH-DHEA or CS was observed regardless of source of enzyme.

DISCUSSION

Dehydroepiandrosterone (DHEA), as its sulfated form, is the most abundant adrenal steroid in the circulation of young adult humans and is secreted concomitantly with glucocorticoids (GC) from the adrenal. DHEA has been proposed to be involved in maintenance of bone, muscle, skin and brain cells, in modification of immune responses, inflammation, lipid and carbohydrate metabolism, in alteration of chemical carcinogenesis, in causing neuroprotective and memory enhancing effects and in ameliorating the deleterious effects of GC [16-19]. DHEA may exert its pleiotropic effects following its conversion into several biologically-active metabolites, including 7 α -hydroxy-DHEA (7 α -OH-DHEA), 7 β -hydroxy-DHEA (7 β -OH-DHEA) and 7-oxo-DHEA [11,20]. For example, 7-oxo-DHEA, as well as 7 α - and 7 β -OH-DHEA, have been proposed to have biological actions in rodent and men [21-24] with higher effectiveness than the parent sterol DHEA [25,26].

Previously, we demonstrated that the interconversion of 7-oxidized metabolites of cholesterol and DHEA are catalyzed by the same set of oxidoreductases that interconvert 11-hydroxy- and 11-oxo-GC, the 11 β HSDs [9]. We also purified an NADP⁺-dependent hamster liver microsomal 7-hydroxycholesterol dehydrogenase to near homogeneity which oxidizes both 7 α - and 7 β -hydroxycholesterol, as well as cortisol and CS, at similar maximal velocities [27]. The oxidation of 7-hydroxycholesterol and CS by this purified enzyme was inhibited by CBX. Subsequently, human, rat, and mouse 11 β HSD1 have been reported to possess a 7 β -hydroxycholesterol dehydrogenase activity that allows interconversion between 7-ketocholesterol and 7 β -hydroxycholesterol with similar efficiency as observed for GC [28, 29]. In the current studies, pig kidney mitochondria oxidized 7 α -OH-DHEA in the presence of either NAD⁺ or NADP⁺ at a very low rate (approx. 30% of that seen formed by pig kidney microsomal fractions). Since PKMw contained microsomal NADPH:cytochrome *c* oxidoreductase activity (Table 1), oxidation of 7 α -OH-DHEA by mitochondria may, in part, be due to contamination with microsomal protein. However, PKMc and PKN both converted 7 α -OH-DHEA to 7-oxo-DHEA at similar rates, although PKN showed no cross contamination

by microsomal NADPH:cytochrome *c* oxidoreductase activity. Both PKMc and PKN preferentially oxidized 7 α -OH-DHEA with an activity that was approx. 6 times higher with NAD⁺, than with NADP⁺ (Figure 1). Rat kidney nuclear fractions oxidized 7 α -OH-DHEA at similar rate to rat kidney microsomal protein when incubated with NAD⁺, but displayed very little conversion when incubated with NADP⁺ [9]. Therefore, the enzymes which oxidize 7 α -OH-DHEA in the kidneys of pig and rat differ in their tissue distribution, in organelle localization, in quantity, and in pyridine nucleotide specificity.

Similar to the activity in rat kidney [9], CS, 11 α -hydroxyprogesterone, and 11 β -hydroxyprogesterone inhibited oxidation of 7 α -OH-DHEA by all pig kidney sub-cellular fractions in the presence of either pyridine nucleotide. However, with either of the pyridine nucleotides, PKN and PKMc converted CS to DHC in a much lower rate than that observed for the oxidation of 7 α -OH-DHEA to 7-oxo-DHEA, demonstrating that the V_{max} for 7 α -OH-DHEA oxidation is much larger than that for CS. Furthermore, the oxidation of CS to DHC was inhibited at higher concentrations of CS. This result suggests that 7 α -OH-DHEA oxidation is catalyzed by low affinity/high capacity enzyme(s), while CS is oxidatively metabolized by high affinity/low capacity one(s). The 11 β HSD2 in kidney of different mammals was described to be a high affinity/low capacity enzyme [30]. The oxidation rate of 7 α -OH-DHEA by PKMc and PKN was much higher in presence of NAD⁺ than in with NADP⁺. However, these organelles converted CS to DHC in presence of NAD⁺ at a rate similar to that observed when NADP⁺ was the pyridine nucleotide co-substrate. This further suggests that in the pig kidney, the enzymatic systems oxidizing CS most likely are different than those catalyzing the oxidation of 7 α -OH-DHEA. The similarity in rates of CS oxidation manifested by both fractions and the pyridine nucleotide specificity found herein for the pig kidney is different than the pattern observed with sheep kidney [10]. In sheep kidney, the nuclear fraction oxidized more cortisol in presence of NAD⁺ than with NADP⁺, while the microsomal fraction showed preference for NADP⁺-dependent rather than NAD⁺-dependent conversion. Our finding that CS is oxidized to DHC in presence of either nucleotide does not fit the conclusion in the literature that NAD⁺-dependent microsomal 11 β HSD2 as the sole kidney 11 β HSD that oxidizes 11-hydroxy-GC in kidney [30]. Our results are in agreement with the NAD⁺- and NADP⁺-dependent oxidation reactions of CS found in sheep kidney [10]. The LLC-PK1 cell line derived from the kidney of a normal healthy male pig oxidizes cortisol in the presence of NAD⁺, as well as of NADP⁺, and has been proposed to contain two different forms of 11 β HSD [31]. In renal proximal tubule cells from rat kidney, the presence of an 11 β HSD that uses NADP⁺ for conversion of CS to DHC has been documented [31].

In the present report, we have also shown the presence of a small, but significant NADPH-dependent reductase activity for CS in pig kidney nuclei (84 ± 18 pmol/mg protein/120 min); an activity that may allow it to convert inactive GC to corticosterone or cortisol. This activity was not observed in the microsomal or mitochondrial organelles of pig kidney in the presence of an NADPH-regenerating system. This result suggests that pig kidney nuclei can reduce inactive glucocorticoids to their active form, possibly leading to deleterious effects in that tissue. Increased GC activation in nuclei may have deleterious effects in kidney and on regulation of body water and sodium content, as well as, on blood pressure.

Since GC potently inhibit the oxidation of 7-oxidized metabolites of DHEA in rodents, pigs and humans, they most likely alter its metabolism and further alter the pleiotropic effects of this steroid *in vivo* and may lead to deleterious effects on clinical therapy by DHEA [19]. The data reported herein does not provide any evidence for a possible effect of 7-oxidized-DHEA metabolites on GC oxidative metabolism in kidney. However, DHEA metabolites may affect GC fate in other organs, since DHEA feeding was shown to decrease 11 β HSD1 gene expression in rat liver [32] and to reduce 11 β HSD1 activity in human myoblasts [32]. In addition, estradiol decreased 11 β HSD1 mRNA and protein in rat kidney to undetectable levels, but yet decreased

the NADP⁺-dependent conversion of CS to DHC by less than 50% [34]. In spontaneously hypertensive rats, DHEA-sulfate enhanced activity of 11 β HSD2 in kidney, diminished 11 β HSD1 action in liver and therefore increased the ratio of DHC/CS in plasma [35]. Since our previous work has demonstrated significant species differences in DHEA metabolism in rat, pig and human liver [9], these studies demonstrate that pig kidney 11 β -HSDs that catalyze oxidative metabolism of these sterols also differ from those observed in the rat. Additional studies are required to assess the role of DHEA metabolite oxidation and their possible interaction with GC processing in the human kidney and the clinical implications of this interaction. If pig and human HSDs are distributed in similar manner, the pig may serve as a superior model for human clinical studies of sterol action.

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
 PKMc, pig kidney microsomal fractions
 PKMw, pig kidney mitochondrial fractions
 PKN, pig kidney nuclei

REFERENCES

1. Blum A, Raum A, Maser E. *Biochemistry* 2003;42:4108–4117. [PubMed: 12680765]
2. Sandeep TC, Walker BR. *Trends Endocrinol. Metab* 2001;12:446–453. [PubMed: 11701343]
3. Atanasov AG, Nashev LG, Schweizer RA, Frick C, Odermatt A. *FEBS Lett* 2004;571:129–133. [PubMed: 15280030]
4. Banhegyi G, Benedetti A, Fulceri R, Senesi S. *J. Biol. Chem* 2004;279:27017–27021. [PubMed: 15090536]
5. White PC, Mune T, Agarwal AK. *Endocrine Rev* 1997;18:135–156. [PubMed: 9034789]
6. Gomez-Sanchez EP, Cox D, Foecking M, Ganjam V, Gomez-Sanchez CE. *Steroids* 1996;61:110–115. [PubMed: 8852827]
7. Bujalska I, Shimojo M, Howie A, Stewart PM. *Steroids* 1997;62:77–82. [PubMed: 9029719]
8. Shimojo M, Ricketts ML, Petrelli MD, Moradi P, Johnson GD, Bradwell AR, Hewison M, Howie AJ, Stewart PM. *Endocrinology* 1997;138:1305–1311. [PubMed: 9048640]
9. Robinson B, Michael KK, Ripp SL, Winters SJ, Prough RA. *Arch. Biochem. Biophys* 2003;412:251–258. [PubMed: 12667489]
10. Gomez-Sanchez EP, Ganjam V, Chen YJ, Cox DL, Zhou MY, Thanigaraj S, Gomez-Sanchez CE. *Steroids* 1997;62:444–450. [PubMed: 9178432]
11. Fitzpatrick JL, Ripp SL, Smith NB, Pierce WM, Prough RA. *Arch. Biochem. Biophys* 2001;389:278–287. [PubMed: 11339818]
12. Bicer I, Aksu K, Parildar Z, Tanyalcin T, Doganavsargil E, Kutay FZ. *Rheumatol. Int* 2003;23:221–225. [PubMed: 14504913]
13. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. *Anal. Biochem* 1985;150:76–85. [PubMed: 3843705]
14. Matsubara T, Prough RA, Burke MD, Estabrook RW. *Cancer Res* 1974;34:2194–2203.

15. King, TE. *Methods in Enzymology*. Estabrook, RW.; Pullman, AME., editors. 10. Acad. Press; New York: 1967. p. 215-225.
16. Ben-Nathan D, Lustig S, Kobiler D, Danenberg HD, Lupu E, Feuerstein G. *J. Med. Virol* 1992;38:159–166. [PubMed: 1287129]
17. Lapchak PA, Araujo DM. *Int. Rev. Neurobiol* 2001;46:379–397. [PubMed: 11599307]
18. Lee KS, Oh KY, Kim BC. *J. Dermatol. Sci* 2000;23:103–110. [PubMed: 10808127]
19. Robinson B, Cutolo M. *Rheumatology (oxford)* 1999;38:488–495. [PubMed: 10402066]
20. Marwah A, Marwah P, Lardy H. *J. Chromatography B* 2002;767:285–299.
21. Hampl R, Lapcik O, Hill M, Klak J, Kasal A, Novacek A, Sterzl I, Sterzl J, Starka L. *Physiol. Res* 2000;49(Suppl 1):S107–S112. [PubMed: 10984079]
22. Morfin R, Starka L. *Int. Rev. Neurobiol* 2001;46:79–95. [PubMed: 11599312]
23. Shi J, Schulze S, Lardy HA. *Steroids* 2000;65:124–129. [PubMed: 10699590]
24. Schipper L, Spee B, Rothuizen J, Woutersen van Nijnanten F, Fink Gremmels J. *Biochim. Biophys. Acta* 2004;1688:68–77. [PubMed: 14732482]
25. Bobyleva V, Bellei M, Kneer N, Lardy H. *Arch. Biochem. Biophys* 1997;341:122–128. [PubMed: 9143361]
26. Lardy H, Kneer N, Wei Y, Partridge B, Marwah P. *Steroids* 1999;64:497.
27. Song W W, Chen J, Dean WL, Redinger RN, Prough RA. *J. Biol. Chem* 1998;273:16223–16228. [PubMed: 9632680]
28. Hult M, Elleby B, Shafqat N, Svensson S, Rane A, Jornvall H, Abrahmsen L, Oppermann U. *Cell. Mol. Life Sci* 2004;61:992–999. [PubMed: 15095019]
29. Schweizer RA, Zurcher M, Balazs Z, Dick B, Odermatt A. *J. Biol. Chem* 2004;279:18415–18424. [PubMed: 14973125]
30. Gu S, Ripp SL, Prough RA, Geoghegan TE. *Mol. Pharmacol* 2003;63:722–731. [PubMed: 12606783]
31. Brem AS, Bina RB, Fitzpatrick C, King T, Tang SS, Ingelfinger JR. *Proc. Soc. Exp. Biol. Med* 1999;221:111–117. [PubMed: 10352121]
32. Stewart PM, Krozowski ZS. *Vitam. Horm* 1999;57:249–324. [PubMed: 10232052]
33. Whorwood CB, Donovan SJ, Wood PJ, Phillips DI. *J. Clin. Endocrinol. Metab* 2001;86:2296–2308. [PubMed: 11344242]
34. Gomez-Sanchez EP, Ganjam V, Chen YJ, Liu Y, Zhou MY, Toroslu C, Romero DG, Hughson MD, de Rodriguez D, Gomez-Sanchez CE. *Am. J. Physiol. Endocrinol. Metab* 2003;285:E272–E279. [PubMed: 12700160]
35. Homma M, Onodera T, Hirabatake M, Oka K, Kanazawa M, Miwa T, Hayashi T. *J. Pharm. Pharmacol* 1998;50:1139–1145. [PubMed: 9821661]

Figure 1.

Time course of conversion of 7 α -OH-DHEA to 7-oxo-DHEA by pig kidney nuclei (PKN), mitochondria (PKMw) and microsomes (PKMc) fractions. The fractions were incubated with 7 α -OH-DHEA (50 μ M) and either 1 mM β -NADP⁺ or 1mM β -NAD⁺ as pyridine nucleotide co-substrate. The enzyme activity was determined as described in the Methods section. The results are expressed as the average enzyme activity (nmol/mg protein) $\pm$ SEM (n=6).

Figure 2.

The effect of corticosterone (CS) on the rate of conversion of 7α -OH-DHEA to 7-oxo-DHEA by pig kidney nuclei (PKN), mitochondria (PKMw) and microsomes (PKMc) fractions. The fractions were incubated with 7α -OH-DHEA ($50 \mu\text{M}$) and either $1 \text{ mM } \beta\text{-NADP}^+$ or $1 \text{ mM } \beta\text{-NAD}^+$ as pyridine nucleotide co-substrate. Ethanol or CS ($10 \mu\text{l}$ in ethanol) was added to the incubation medium to attain concentration of $50 \mu\text{M}$, prior to addition of the subcellular fraction and 7α -OH-DHEA. The enzyme activity was determined as described in the Methods section. The results are expressed as the average enzyme activity ($\text{nmol/mg protein/120 min}$) $\pm$ SEM ($n=6$).

Figure 3.

Time course of conversion of CS to DHC by pig kidney microsomal fractions (PKMc). The fractions were incubated with CS (2 μ M) and either 1 mM β -NADP⁺ or 1mM β -NAD⁺ as pyridine nucleotide co-substrate. The enzyme activity was determined as described in the Methods section. The results are expressed as the average enzyme activity (nmol/mg protein) $\pm$ SEM (n=6).

Figure 4.

The effect of DHC, 7α -OH-DHEA or 7-oxo-DHEA on the conversion of CS to DHC by PKMc (a) or PKN (b) protein fractions. The fractions were incubated with CS (2 μ M) and either 1 mM β -NADP⁺ or 1mM β -NAD⁺ as pyridine nucleotide co-substrate. Ethanol, DHC, 7α -OH-DHEA or 7-oxo-DHEA (10 μ l in ethanol, 50 μ M final concentration) was added to the incubation medium to attain sterol concentration of 50 μ M, prior to addition of the subcellular fraction and CS. The enzyme activity for 120 min was determined as described in the Methods section. The results are expressed as the average enzyme activity (nmol/mg protein/120 min) $\pm$ SEM (n=6).

Figure 5.

The effect of DHC or 7-oxo-DHEA on the rate of conversion of 7 α -OH-DHEA to 7-oxo-DHEA by PKMc or PKN fractions. The fractions were incubated with 7 α -OH-DHEA (50 μ M) and either 1 mM β -NADP⁺ or 1 mM β -NAD⁺ as pyridine nucleotide co-substrate. Ethanol, DHC, or 7-oxo-DHEA (10 μ l in ethanol, 50 μ M final concentration) was added to the incubation medium to attain sterol concentration of 50 μ M, prior to addition of the subcellular fraction and 7 α -OH-DHEA. The enzyme activity for 120 min was determined as described in the Methods section. The results are expressed as the average enzyme activity (nmol/mg protein/120 min) $\pm$ SEM (n=6).

Table 1

Enzyme assay for cross-contamination of porcine kidney nuclear, microsomal, and mitochondrial membrane fractions. After the various tissue fractions were obtained by differential sedimentation, the NADPH:cytochrome *c* and succinate:cytochrome *c* oxidoreductase activities were determined as described in Methods. The results shown are the average of triplicate assays for three different preparations of pig kidney tissue fractions.

Tissue Fraction	NADPH:cytochrome <i>c</i> oxidoreductase activity (nmol/	Succinate:cytochrome <i>c</i> oxidoreductase activity (nmol/
	min/mg)	min/mg)
Nuclei	0.028 ± 0.049	0.76 ± 0.21
Mitochondria	5.41 ± 1.48	25.8 ± 3.4
Microsomes	14.0 ± 2.5	6.64 ± 0.67

Table 2

The effects of carbenoxolone (CBX) and 11 α - or 11 β -hydroxy-progesterone (11 α - or 11 β -OH-P) on the rate of conversion of CS to DHC by pig kidney microsomes and nuclei. The various fractions were incubated with either 1 mM β -NADP⁺ or 1mM β -NAD⁺ as co-substrate, 2 μ M CS, and inhibitors CBX (2 μ M) and 11 α - or 11 β -OH-PRO (50 μ M). The enzyme activity was determined as described in the Methods section. The results are expressed as the average enzyme activity (pmol/mg protein/hr) $\pm$ SEM (n=6)

Subcellular Fraction	Pyridine Nucleotide	Control Activity (pmol/mg/hr)	% Inhibition		
			CBX	11 α -OH-PRO	11 β -OH-PRO
PKN	NAD	698 $\pm$ 21	92 $\pm$ 1	100 $\pm$ 0	100 $\pm$ 0
PKN	NADP	535 $\pm$ 44	98 $\pm$ 1	100 $\pm$ 0	100 $\pm$ 0
PKMc	NAD	749 $\pm$ 65	89 $\pm$ 1	93 $\pm$ 2	96 $\pm$ 2
PKMc	NADP	704 $\pm$ 77	86 $\pm$ 2	97 $\pm$ 1	98 $\pm$ 0

Table 3

Pyridine nucleotide specificity for conversion of CS to DHC and 7 α -OH-DHEA to 7-oxo-DHEA by pig kidney microsomes and nuclei. The fractions (2 and 1 mg/ml respectively) are incubated with either no pyridine nucleotide, 1 mM β -NADP⁺, 1mM β -NAD⁺, or 1 mM β -NADP⁺ plus 1mM β -NAD⁺, and either 2 μ M CS or 50 μ M 7 α -OH-DHEA as sterol substrates. The enzyme activity was determined as described in the Methods section. The results are expressed as the average enzyme activity (pmol/mg protein/hr) $\pm$ SEM (n=6).

Subcellular Fraction	Substrate	Pyridine Nucleotide (1mM)			
		NAD ⁺	NADP ⁺	NAD + NADP	None
PKN	CS	720 $\pm$ 20	500 $\pm$ 40	710 $\pm$ 30	13 $\pm$ 9
PKMc	CS	750 $\pm$ 38	710 $\pm$ 74	730 $\pm$ 60	7 $\pm$ 5
PKN	7 α -OH-DHEA	5853 $\pm$ 244	1011 $\pm$ 214	5270 $\pm$ 181	19 $\pm$ 15
PKMc	7 α -OH-DHEA	5790 $\pm$ 200	1004 $\pm$ 190	5781 $\pm$ 208	8 $\pm$ 6