Regulation of Human 3β -Hydroxysteroid Dehydrogenase Type 2 by Adrenal Corticosteroids and Product-Feedback by Androstenedione in Human Adrenarche

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ABSTRACT

In human adrenarche during childhood, the secretion of dehydroepiandrosterone (DHEA) from the adrenal gland increases due to its increased synthesis and/or decreased metabolism. DHEA is synthesized by 17α -hydroxylase/ $17,20$ -lyase, and is metabolized by 3β -hydroxysteroid dehydrogenase type 2 (3β HSD2). In this study, the inhibition of purified human 3β HSD2 by the adrenal steroids, androstenedione, cortisone, and cortisol, was investigated and related to changes in secondary enzyme structure. Solubilized, purified 3β HSD2 was inhibited competitively by androstenedione with high affinity, by cortisone at lower affinity, and by cortisol only at very high, nonphysiologic levels. When purified 3β HSD2 was bound to lipid vesicles, the

competitive K_i values for androstenedione and cortisone were slightly decreased, and the K_i value of cortisol was decreased 2.5-fold, although still at a nonphysiologic level. The circular dichroism spectrum that measured 3β HSD2 secondary structure was significantly altered by the binding of cortisol, but not by androstenedione and cortisone. Our import studies show that 3β HSD2 binds in the intermitochondrial space as a membrane-associated protein. Androstenedione inhibits purified 3β HSD2 at physiologic levels, but similar actions for cortisol and cortisone are not supported. In summary, our results have clarified the mechanisms for limiting the metabolism of DHEA during human adrenarche.

Introduction

Adrenarche is the process of maturation of the adrenal zona reticularis, resulting in increased secretion of the adrenal androgen precursors dehydroepiandrosterone (DHEA) and the sulfate ester, DHEA-sulfate (DHEAS) (Lashansky et al., 1991; Miller and Auchus, 2011). Premature adrenarche is defined as increased secretion of adrenal androgens before the age of 8 years in girls and 9 years in boys, and the concurrent presence of signs of androgen action including adult-type body odor, oily skin, and pubic hair growth (Idkowiak et al., 2011). Premature adrenarche may represent a forerunner condition for polycystic ovarian syndrome, insulin resistance, or metabolic

syndrome (Ibanez et al., 2009; Diamanti-Kandarakis and Dunaif, 2012). The exact trigger mechanisms for adrenarche remain unknown, but must result from increased synthesis and/or decreased metabolism of DHEA, the key adrenal steroid precursor of the sex hormones later in human development.

In humans, adrenal synthesis of DHEA requires the enzymes 17α -hydroxylase and $17,20$ -lyase (both components of cytochrome P450 c17, encoded by *CYP17A1*). Metabolism of DHEA to androstenedione is performed by 3β -hydroxysteroid dehydrogenase 2 (3β HSD2), and to DHEAS by DHEA-sulfotransferase (*SULT2A1*) (Rainey et al., 2002). Activation of $17,20$ -lyase is postulated to play a role in triggering adrenarche (Miller, 2009). Phosphorylation of cytochrome P450 c17 enhances $17,20$ -lyase activity (Zhang et al., 1995; Geller et al., 1997) and may be mediated by p38 α (Tee and Miller, 2013). Whether p38 α activity increases during adrenarche is not known.

A reduction in 3β HSD2 activity is also postulated to play a role in the onset of adrenarche (Rainey and Nakamura, 2008). 3β HSD2 content in the zona reticularis decreases following adrenarche (Endoh et al., 1996; Gell et al., 1998). Further, we recently reported that cortisol and cortisone inhibit 3β HSD2 activity and stimulate DHEA and DHEAS production in human adrenocortical NCI-H295R cells, and

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ABBREVIATIONS: CD, circular dichroism; CFS, cell-free transcription-translation system; DHEA, dehydroepiandrosterone; DHEAS, DHEA-sulfate; 3β HSD2, 3β -hydroxysteroid dehydrogenase 2; HT, hydroxyapatite; IMS, intermitochondrial space; OMM, outer mitochondrial membrane; PC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; PK, proteinase K; Tm, thermal melting.

cortisol competitively inhibits 3β HSD2 activity expressed in transfected COS-7 cells (Topor et al., 2011). The effective concentrations of cortisol in these studies (10–500 μ M) are consistent with an allosteric rather than transcriptional mechanism of action. For this reason, we performed the current study, in which we investigated the inhibition of purified human 3β HSD2 by the intra-adrenal steroids cortisol, cortisone, and androstenedione. We have previously reported product inhibition kinetics with androstenedione using purified human placental 3β HSD1 (Thomas et al., 1989). The current investigation is the first to report the inhibition kinetics of purified human adrenal 3β HSD2 by androstenedione, and suggests a role for androstenedione in keeping 3β HSD2 activity low during human adrenarche. Our recent studies on the mechanisms of the mitochondrial localization of human 3β HSD2 also led us to compare the differential effects of liposomal versus solubilized enzyme.

Our current study measures the inhibition of solubilized and liposome-bound preparations of purified human 3β HSD2 by androstenedione, cortisol, and cortisone. Analyses of 3β HSD2 bound to steroid inhibitors in different protein conformations are examined using analysis by far-UV circular dichroism. 3β HSD2 metabolic activity studies are performed in the presence or absence of the phospholipid used to produce the liposomes, PC (1-palmitoyl-2-oleoyl-phosphatidyl-choline). Import studies determine how 3β HSD2 binds in the mitochondrial membranes. This investigation clarifies how 3β HSD2 is inhibited by adrenal steroids, which may contribute to the onset of human adrenarche.

Materials and Methods

Dehydroepiandrosterone, cortisone, cortisol, and pyridine nucleotides were purchased from Sigma-Aldrich (St. Louis, MO); 5-androstene-3,17-dione from Steraloids Inc. (Newport, RI); and reagent grade salts, chemicals, and analytical grade solvents from Fisher Scientific Co. (Pittsburgh, PA). Glass-distilled, deionized water was used for all aqueous solutions. MA-10 cells were obtained from the University of California–San Francisco cell bank. The University of California–San Francisco regularly authenticates and checks for mycoplasma contamination prior to shipment.

Expression and Purification of Human 3β HSD2. 3β HSD2 cDNA was introduced into baculovirus as previously described (Thomas et al., 1998). The recombinant baculovirus was incubated with 2.25×10^9 Sf9 (American Type Culture Collection, Manassas, VA) cells in 1.5 l at a multiplicity of infection of 10. To confirm expression of 3β HSD2, proteins from the Sf9 cells were separated by SDS-PAGE (12%), probed with our anti- 3β HSD2 polyclonal antibody (Pawlak et al., 2011), and detected using the West Pico Western blotting system (Pierce, IL). The expressed enzyme was purified from a 100,000g pellet of Sf9 cells by our published method (Thomas et al., 1989, 2002), using Igepal CO-720 (Sigma-Aldrich). SDS-PAGE (12%) of the purified enzyme resulted in a single band at 42 kDa that comigrated with the control 3β HSD2 enzyme. A high critical micelle concentration detergent with no UV absorbance, Cymal-5 (Anatrace, Inc., Maumee, OH), was exchanged for Igepal using hydroxyapatite chromatography. The 3β HSD2 fraction pool from the DEAE column was applied to the hydroxyapatite (HT) column (1 mg protein/ml packed gel), washed with 3.5 column volumes of 0.025 M potassium phosphate (pH 7.5), 20% glycerol, 0.1 mM EDTA, 0.01 M NAD^+ , 1.8 mM Cymal-5, and then eluted with 0.30 M potassium phosphate (pH 7.5), 20% glycerol, 0.1 mM EDTA, 0.01 M NAD^+ , and 1.8 mM Cymal-5. After elution from the HT column (Fig. 1A) and SDS-PAGE analysis of purity (Fig. 1B), HT fractions with peak 3β HSD2 activity were pooled and found to be free of Igepal CO-720 based on its absorbance at 280 nm. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard. During enzyme purification, the isomerase activity of 3β HSD2 was measured by the initial absorbance increase at 241 nm due to androstenedione formation from the intermediate substrate, 5-androstene-3,17-dione (100 μ M), as a function of time. Blank assays (zero enzyme, zero substrate) ensured that specific isomerase activity was measured as opposed to nonenzymatic, “spontaneous” isomerization. Changes in absorbance were measured with a Varian Cary 300 (Sugar Land, TX) spectrophotometer.

Kinetic Studies. Michaelis-Menten kinetic constants for the 3β HSD2 substrate were determined for the purified wild-type 3β HSD2 enzyme in incubations containing DHEA (2–100 μ M) plus NAD^+ (0.2 mM) and purified enzyme (0.04 mg) at 27°C in 0.02 M potassium phosphate, pH 7.4. The slope of the initial linear increase in absorbance at 340 nm/min (due to NADH production) was used to determine 3β HSD2 activity (Thomas et al., 1998). Changes in absorbance were measured with a Varian Cary 300 recording spectrophotometer. The Michaelis-Menten constants (K_m , V_{max}) were calculated from Lineweaver-Burke (1/S versus 1/V) plots, then verified by Hanes-Woolf (S versus S/V) plots and nonlinear regression analysis (GraphPad Prism 6; GraphPad Software, San Diego, CA).

Fig. 1. Purification and analysis of the purified 3β HSD2. (A) The 3β HSD2 pool from the DEAE column was applied to an HT column for detergent exchange, washed, eluted in fractions 21–27, and plotted as total 3β HSD2 activity (nmol NADH/min, ●, solid line), total protein (mg, ▲, broken line). (B) SDS-PAGE (7.5%) of the purified 3β HSD2 in fractions from the HT column. The HT column was used as the final purification step and to exchange detergents as described in *Materials and Methods*. Each lane was overloaded with 4.0 μ g of purified protein from the HT column fraction, and the bands were visualized by Coomassie Blue staining.

Inhibition constants (K_i) were determined for the inhibition of 3 β HSD2 activity by cortisol, cortisone, and androstenedione using appropriate conditions based on substrate K_m values. For solubilized 3 β HSD2, incubations at 27°C contained subsaturating concentrations of substrate DHEA (8.0 or 20.0 μ M; DHEA K_m = 23 μ M for pure, solubilized 3 β HSD2), NAD⁺ (0.2 mM), purified 3 β HSD2 enzyme (0.04 mg), and cortisol (0–600 μ M), cortisone (0–200 μ M), or androstenedione (0–10.0 μ M) in 0.02 M potassium phosphate buffer, pH 7.4. For liposome-bound 3 β HSD2, similar incubations contained substrate DHEA (6.0 or 10.0 μ M; DHEA K_m = 13 μ M for pure, liposome-bound 3 β HSD2) and cortisol (0–400 μ M), cortisone (0–200 μ M), or androstenedione (0–10.0 μ M). Dixon analysis (I versus 1/V) and nonlinear regression analyses were used to determine the type or mode of inhibition (competitive, noncompetitive, uncompetitive) and calculate the inhibition constant (K_i) values (Segel, 1993). The Dixon plot is widely used to characterize enzyme inhibition kinetics. This method uses an optimized range of four to six inhibitor concentrations compared with nonlinear regression analysis that uses just two to three inhibitor concentrations. With Dixon plots, the mode of enzyme inhibition is visually apparent, unlike nonlinear regression analysis. Both methods yielded very similar K_i values, which represent the inhibitor concentration that reduces maximal enzyme activity by 50% and is considered a measure of the affinity of the enzyme for the inhibitor. A decrease in K_i indicates an increase in affinity (Segel, 1993).

Vesicle Preparation. The phospholipid (PC; 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) was dissolved in chloroform and dried under nitrogen gas. Samples were then hydrated with 10 mM Na₂HPO₄ buffer (pH 7.4), frozen in liquid nitrogen, and thawed on ice. This cycle was repeated five times to make large multilamellar vesicles. Next these large multilamellar vesicles were converted to small unilamellar vesicles using high-pressure excursion technique by extrusion at room temperature through a polycarbonate membrane with 100-nm pores as described previously (Bell et al., 1996; Dowhan and Bogdanov, 2009). PC vesicles were chosen based on obtaining liposomal 3 β HSD2 activity that was similar to the solubilized enzyme compared with vesicles made from other lipids that produced much lower enzyme activity.

Isolation and Purification of Mitochondria. Mitochondria were obtained from MA-10 cells, NCI H295R cells, and as a control, from pig adrenal glands. The pig adrenals were excised immediately after animal sacrifice at the Department of Animal Sciences, University of Florida (Gainesville, FL). The adrenal tissues were diced in mitochondrial isolation buffer (250 mM sucrose, 10 mM HEPES, 1 mM EGTA, pH 7.4) while the cell lines were washed with phosphate-buffered saline two times and then incubated with 1 mM HEPES (pH 7.4) for 30 minutes at 4°C. Next, tissues and cells were homogenized in a hand-held all-glass Dounce homogenizer with 40 gentle up-and-down strokes, and the cell debris removed by centrifugation at 3500g for 10 minutes. The supernatant containing the mitochondrial fraction was purified by differential centrifugation following a previously reported procedure (Bose et al., 2007, 2008), and the pellet was washed and then resuspended in an energy regeneration buffer [125 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 10 mM NaH₂PO₄, 10 mM isocitrate, 1.0 mM ATP, 1.0 mM NADP, 0.1 mM ADP, and 25 mM HEPES (pH 7.4)] prior to storage either at –86°C or in liquid nitrogen.

Metabolic Conversion Assays. Isolated mitochondria (300 μ g) were incubated and then aliquoted into 10 fractions (30 μ g each) for independent metabolic activity with and without purified 3 β HSD2 (2 μ g) in 0.02 M potassium phosphate, pH 7.4, for the conversion of [³H]pregnenolone to [³H]progesterone. Three million cpm of [³H]pregnenolone was used for each reaction, and chased with 30 μ g of cold unlabeled progesterone at the beginning to initiate the reaction. The reaction was initiated by addition of NAD⁺ and incubated at 37°C for 4 hours in a shaking water bath. To investigate the effect of PC in the metabolic conversion of 3 β HSD2, we determined the amount of mitochondria required to generate endogenous metabolic conversion. Trilostane (5 pmol) was used as a control to verify 3 β HSD2 by

inhibition of activity. Then we added PC with a 200-fold difference in lipid concentration (2, 4, 10, 100, 200, and 400 μ g of total PC). For both reactions, the steroids were extracted with ether/acetone (9:1 v/v), and equal amounts of a cold pregnenolone-progesterone (50:50; Sigma-Aldrich) mixture in CH₂Cl₂ was added as a carrier. The extracts were concentrated under a stream of nitrogen or air and then separated by thin-layer chromatography (Whatman, Shrewsbury, MA) using a chloroform/ethyl acetate (3:1) mobile phase.

Thermal Denaturation Studies. Melting curves were obtained by measuring the circular dichroism (CD) signal at 222 nm as a function of temperature, which ranged from 4 to 90°C. The temperature was increased 0.5°C per minute. The additive effects of denaturation were determined by the addition of lipids of various compositions with 3 β HSD2 after equilibration for 2 hours.

Circular Dichroism. CD experiments in the far-UV region (185–250 nm) were carried out using a 2.0-mm path-length quartz cuvette at 20°C in a Jasco J-815 spectropolarimeter (Easton, MD) equipped with a Peltier temperature-controlled cell holder. The instrument was purged with a continuous flow of nitrogen at 10 l/min to reduce the maximum signal-to-noise ratio. Purified 3 β HSD2 was equilibrated in NaH₂PO₄ buffers at pH 7.4, and the CD spectra recorded in the far-UV region are presented without mathematical smoothing. Secondary structural analysis was carried out using the CD-Pro software (Fort Collins, CO) (Sreerama and Woody, 1993, 2000, 2004; Sreerama et al., 2000; Prasad et al., 2012) to determine the relative proportions of α -helix and β -sheet as a function of lipid concentration. To determine the effect of steroids on the conformation of 3 β HSD2 (20 μ g), we made a stock solution of androstenedione (10 mM), cortisone (10 mM), and cortisol (15 mM) in 4% ethanol, which was diluted to the final concentrations (see the Fig. 4 legend) with the working buffer 10 mM NaH₂PO₄, pH 7.4. Prior to studying the conformational changes, we mixed the protein and steroids at the desired concentration and then incubated at 20°C for 30 minutes. For lipid vesicle effect determinations, the PC vesicles were mixed with a fixed concentration of 3 β HSD2 at pH 7.4 (Fig. 3, B and C). In a separate set of experiments (Fig. 3D), purified wild-type 3 β HSD2 was equilibrated in 10 mM NaH₂PO₄ at pH 7.4 with two concentrations of PC lipid (50 or 250 μ g). For each set of measurements, the appropriate buffer blank was subtracted from each spectrum, and θ at 222 nm was plotted with respect to temperature or vesicle composition.

Protein Import into Mitochondria. To synthesize [³⁵S]methionine-labeled proteins, the cDNAs for 3 β HSD2 and the Tim50 fusion were subcloned into the SP6 vector (Bose et al., 2002b) and proteins were synthesized in a cell-free-linked transcription-translation system (CFS) in the presence of [³⁵S]methionine using TNT-rabbit reticulocyte (Promega, Madison, WI). Ribosomes and associated incompletely translated polypeptide chains were removed by centrifugation at 150,000g for 15 minutes at 4°C (Schwartz and Matouschek, 1999). For all protein import experiments, a 100- μ l mixture of isolated mitochondria (100 μ g) and synthesized protein was incubated in a 26°C water bath with or without proteinase K (PK), and the import reaction terminated by the addition of 1 mM *m*-chlorocarbonylcyanide phenylhydrazide (uncoupler of oxidative phosphorylation) and an equal volume of boiling 2 $\times$ SDS sample buffer. The mitochondrial imported fractions were characterized by limited proteolysis with proteinase K, and were then completely proteolyzed by the addition of 0.5% Triton X-100. The presence of the same amount of mitochondria applied to all experimental procedures was confirmed by western blotting with appropriate antibodies. The import reactions were analyzed by electrophoresing the samples through SDS-polyacrylamide gels, fixing the gels in methanol/acetic acid (40:10), and then drying and exposing gels to a phosphorimager screen. Using our CFS, ³⁵S-labeled 3 β HSD2 was imported into the isolated mitochondria of NCI-H295R cells, a human pluripotent adrenocortical cell line, and the imported pellet fraction (P) in the mitochondria was separated from the unimported supernatant (S) fraction by centrifugation. A fraction of the 3 β HSD2 was extracted from the nonintegrated supernatant (S)

fraction after incubation of Na_2CO_3 (pH 11.5) followed by centrifugation at 13,000g for 30 minutes at 4°C. The Tim50-3 β HSD2 construct [(1-50)Tim50-3 β HSD2] was also imported into the mitochondria and proteolyzed in the presence of PK with and without the addition of 0.5% Triton X-100. Western blot with the VDAC2 antibody recognizes VDAC2, a mitochondrial voltage-dependent anion channel 2 protein in the outer mitochondrial membrane (OMM) that is used as a marker protein (32 kDa).

Results

Inhibition of 3 β HSD2 by Androstenedione, Cortisone, and Cortisol. Measurement of enzyme kinetics, as shown in Table 1, revealed that androstenedione is a much better inhibitor of solubilized, purified 3 β HSD2 than either cortisone (8-fold lower affinity) or cortisol (80-fold lower affinity) based on the measured K_i values. Androstenedione and cortisone inhibit purified 3 β HSD2 with a competitive mode for both the solubilized and the liposome-bound enzymes. The K_i values for androstenedione and cortisone are 1.4- and 1.6-fold lower for liposome-bound 3 β HSD2 than for solubilized 3 β HSD2, respectively. However, cortisol inhibits both the solubilized and liposome-bound 3 β HSD2 in an uncompetitive mode. Furthermore, cortisol inhibits the liposome-bound 3 β HSD2 with a 2.5-fold lower K_i value (or higher affinity) compared with its extremely low-affinity inhibition of solubilized 3 β HSD2 (Table 1). The competitive modes of inhibition of pure and liposome-bound 3 β HSD2 by androstenedione and cortisone are clearly shown by the intersecting Dixon plots in Fig. 2, A and B, and C and D, respectively. The uncompetitive modes of inhibition of pure and liposome-bound 3 β HSD2 by cortisol are shown by the parallel Dixon plots in Fig. 2, E and F.

Metabolic Activity of Liposomal 3 β HSD2. Our kinetic results (Fig. 2; Table 1) suggest that lipid vesicles might influence enzyme conformation. We first determined the influence of lipid membrane on enzyme activity by incubating baculovirus-purified 3 β HSD2 with isolated steroidogenic mitochondria and lipid vesicles. Measurable endogenous activity was low with 30 μg of mitochondria in the absence of PC, and increased 3.4-fold after addition of exogenous 3 β HSD2. The addition of PC lipid in concentrations up to 100 μg produced maximal 3 β HSD2 activity (Fig. 3A). The much lower endogenous mitochondrial 3 β HSD2 activity was also stimulated by PC, but the PC effect was maximal at 20 μg without exogenous 3 β HSD2 (Fig. 3B). These enzyme activity data that are shown as thin-layer chromatography spots in Fig. 3, A and B, are plotted quantitatively in Fig. 3C. This suggests that a lipid environment stimulates 3 β HSD2

activity, likely by changing enzyme topology. The fatty acid PC contains a saturated chain in the sn-1 position and an unsaturated chain in the sn-2 position, mimicking mammalian phospholipid composition. Because of the ring system and alkyl chain of the sterol, as well as the glycerol backbone, the choline head group and the acyl chains possibly interact with 3 β HSD2 to activate it. This interaction with 3 β HSD2 is essential for increased activity either at the intermitochondrial space (IMS) or through a proton gradient (Prasad et al., 2012) from the matrix to the IMS. An excess of PC had no further effect on enzyme activity, presumably because all 3 β HSD2 had been fully titrated.

Enzyme Conformation Induced by Lipid Vesicles.

Studies have indicated that the environment, including lipid composition, can often influence protein conformation and folding of 3 β HSD2 (Gohil and Greenberg, 2009). Since the aforementioned experiment showed an interaction between 3 β HSD2 and lipid membranes, we next tested whether lipid membranes stimulate the protein to adopt a conformation suitable for this interaction. Lipid influence may be particularly pertinent for 3 β HSD2 as this protein resides in the inner mitochondrial membrane space (Pawlak et al., 2011). CD spectroscopy can easily identify secondary structural characteristics: the presence of minima near 198 nm indicates random coils, at 208 and 222 nm indicates α -helices, and at 218 nm indicates β -sheets (Bose et al., 2009a,b; Bose, 2011). As the lipid composition changes, flexible domains of the protein bind with the lipid vesicles; thus, an increase in lipid concentration can further alter conformation. In the presence of increasing concentrations of PC, from 50 to 250 μg , 3 β HSD2 fully retained the α -helical character it exhibited in the absence of lipid (Fig. 3D). After the addition of 50 μg of PC, the overall conformation was unchanged, but the decrease in minima both at 208 and 222 nm is suggestive of increased stability. The addition of a 5-fold increase in concentration of the same lipid membrane (250 μg of PC) resulted in an insignificant change in ellipticity at the $\pi-\pi^*$ position, 208 nm (Fig. 3D), whereas the ellipticity at the 222 nm, n-p* position remains unchanged, suggesting that the protein bound to PC with the polar charged group, leading to protein stability, because of increased content of helical conformation. To determine if the degree of reduction in ellipticity was indeed due to the charged vesicles, we measured the helical content after addition of increasing concentrations of PC. Figure 3E, inset, shows the analysis of the change in protein conformation. We determined that the wild-type 3 β HSD2 protein consisted of approximately 32% α -helix and 25% β -sheet, with the remainder as random coils or turns.

TABLE 1

Inhibition of purified, solubilized human 3 β HSD2 compared with purified, liposomal 3 β HSD2 by androstenedione, cortisone, and cortisol

Data are presented as $\mu\text{M} \pm \text{S.D.}$ For purified, solubilized 3 β HSD2, incubations at 27°C contained subsaturating concentrations of substrate DHEA (8.0 or 20.0 μM ; DHEA $K_m = 23 \mu\text{M}$ for pure, soluble 3 β HSD2), NAD^+ (0.2 mM), purified 3 β HSD2 enzyme (0.04 mg), and cortisol (0–600 μM), cortisone (0–200 μM), or androstenedione (0–7.5 μM) in 0.02 M potassium phosphate buffer, pH 7.4. For liposome-bound 3 β HSD2, similar incubations contained substrate DHEA (6.0 or 10.0 μM ; DHEA $K_m = 13 \mu\text{M}$ for pure, liposome-bound 3 β HSD2) and cortisol (0–400 μM), cortisone (0–100 μM), or androstenedione (0–10.0 μM).

Enzyme	Androstenedione K_i	Cortisone K_i	Cortisol K_i
	μM		
Solubilized 3 β HSD2	6.6 ± 0.4 , competitive	54 ± 2.9 , competitive	542 ± 23 , uncompetitive
Liposomal 3 β HSD2	4.7 ± 0.3 , competitive	34 ± 1.8 , competitive	217 ± 11 , uncompetitive

Fig. 2. Inhibition of solubilized and liposome-bound purified β HSD2 activity by androstenedione, cortisone, and cortisol. (A) Inhibition of solubilized β HSD2 by androstenedione. Incubations at 27°C contained subsaturating concentrations of substrate DHEA [20.0 (●) or 8.0 μ M (◆)]; NAD^+ (0.2 mM); purified, solubilized human β HSD2 enzyme (0.04 mg); and androstenedione (0–7.5 μ M) in 0.02 M potassium phosphate buffer, pH 7.4. (B) Inhibition of liposome-bound β HSD2 by androstenedione. Incubations at 27°C contained subsaturating concentrations of substrate DHEA [10.0 (■) or 6.0 μ M (▲)]; NAD^+ (0.2 mM); purified, liposome-bound human β HSD2 enzyme (0.04 mg); and androstenedione (0–10.0 μ M) in 0.02 M potassium phosphate buffer, pH 7.4. (C) Inhibition of solubilized β HSD2 by cortisone. Incubations at 27°C contained subsaturating concentrations of substrate DHEA [20.0 (●) or 8.0 μ M (◆)]; NAD^+ (0.2 mM); purified, solubilized human β HSD2 enzyme (0.04 mg); and cortisone (0–200 μ M) in 0.02 M potassium phosphate buffer, pH 7.4. (D) Inhibition of liposome-bound β HSD2 by cortisone. Incubations at 27°C contained subsaturating concentrations of substrate DHEA [10.0 (■) or 6.0 μ M (▲)]; NAD^+ (0.2 mM); purified, liposome-bound human β HSD2 enzyme (0.04 mg); and cortisone (0–100 μ M) in 0.02 M potassium phosphate buffer, pH 7.4. (E) Inhibition of solubilized β HSD2 by cortisol. Incubations at 27°C contained subsaturating concentrations of substrate DHEA [20.0 (●) or 8.0 μ M (◆)]; NAD^+ (0.2 mM); purified, solubilized human β HSD2 enzyme (0.04 mg); and cortisol (0–600 μ M) in 0.02 M potassium phosphate buffer, pH 7.4. (F) Inhibition of liposome-bound β HSD2 by cortisol. Incubations at 27°C contained subsaturating concentrations of substrate DHEA [10.0 (■) or 6.0 μ M (▲)]; NAD^+ (0.2 mM); purified, liposome-bound human β HSD2 enzyme (0.04 mg); and cortisol (0–400 μ M) in 0.02 M potassium phosphate buffer, pH 7.4. Each point on the Dixon plots (1/V versus I) represents the mean of duplicate determinations, and the error bars represent standard deviations. K_i values were calculated from the intersection of the Dixon plots obtained for the competitive inhibitors, androstenedione and cortisone. K_i values were calculated from $X_{\text{int}} = -K_i(1 + K_m/S)$ of the plots for uncompetitive inhibition by cortisol. X_{int} , x-axis intercept.

Addition of an equal mixture of PC vesicles up to 50 μ g increased the α -helical content, and did not change further with increase in PC concentration, suggesting that after β HSD2 bind with the vesicles, further availability of the charged residues did not affect the conformation, suggesting that the availability of the sites is saturated.

To further evaluate the stability of β HSD2, we measured thermal melting (T_m) in the presence and absence of lipid membrane. The far-UV CD of β HSD2 at 20°C resulted in minima at 208 and 222 nm, a typical characteristic of the α -helical conformation. The change in ellipticity at 222 nm was measured in temperatures ranging from 4 to 90°C (Fig. 3E) and the derivatives ($d\theta/dT$) plotted as a function of temperature to yield the exact T_m (Fig. 3E, inset). The protein showed ellipticity changes at or above 40°C and complete denaturation at 60°C. The T_m was 42°C in the absence of lipid (Fig. 3E, inset). β HSD2 unfolded in a similar manner in the presence of zwitterionically charged PC vesicles (Fig. 3E). The T_m for these conditions was approximately 42°C in the presence of PC alone. The curves are typical for thermal denaturation. Based on our results, we conclude that lipids help the protein remain associated in a fashion in which the charged residues are oriented with their charged groups

buried, and thus minimally change β HSD2 to promote the association of the protein with the vesicles.

Binding of β HSD2 with Androstenedione, Cortisone, and Cortisol. The CD spectrum of the wild-type β HSD2 protein at pH 7.4 in 10 mM sodium phosphate buffer gave minima in the vicinity of 208 and 222 nm, indicating a predominantly α -helical conformation (Fig. 4A). The concentration used for each steroid was based on the K_i values measured for the β HSD2 (Table 1). The addition of cortisone and androstenedione produced small shifts in the 208–222 nm range in the liposome-bound enzyme, but not in the solubilized enzyme without lipid vesicles (Fig. 4C). However, the addition of cortisol dramatically altered the 208–222 nm absorption in solubilized β HSD2 (Fig. 4B) and induced a less pronounced spectral shift with liposome-bound β HSD2 (Fig. 4D). We have previously observed that the presence of lipids or a chaperone environment stimulates mitochondrial β HSD2 activity (Rajapaksha et al., 2011, 2013b).

Import and Processing of β HSD2 into the IMS. Mitochondrial fractionation of MA-10 cells showed that β HSD2 is localized at the IMS, and is not integrated into the membrane (Prasad et al., 2012). However, it is not known how β HSD2 is imported and translocated into the mitochondria for membrane

Fig. 3. Effect of lipid membrane on 3β HSD2 metabolic activity. (A) Metabolic conversion of accumulated pregnenolone (Prog) to progesterone (Preg) by mitochondria isolated from MA-10 cells incubated in the presence of increasing concentrations of externally added PC. Baseline metabolic conversion was observed with mitochondria (Mito) in the absence of 3β HSD inhibitor, trilostane (Trilo). Conversion was enhanced after the addition of purified 3β HSD2 ($3\beta 2$; 2 μ g) and stimulated further by PC (2, 4, 10, 100, 200, 400 μ g of total PC) with peak activity at 100 μ g PC. The bottom panels are the loading controls of the amount of mitochondria applied in each reaction shown by the western blotting with the indicated antibodies. (B) Similar metabolic conversion with the same amount of mitochondrial protein in the absence of external addition of 3β HSD2 but with the exact same concentrations of lipid as indicated in (A). The bottom panels are the loading control of the amount of mitochondria applied in each reaction shown by the western blotting with the indicated antibodies. (C) The relative amount of progesterone synthesis is plotted on the y-axis, and the PC concentration (PC/ μ g enzyme) is on the x-axis. The relative activity data are indicative of increased synthesis from (A) with added 3β HSD2 and control mitochondrial activity in the absence of purified 3β HSD2 from (B). These data show the quantitative estimation with an increase in synthesis of progesterone after addition of increasing concentration of lipid and 3β HSD2 together [black line with closed square (■)] or with lipid in the absence of 3β HSD2 [red broken line with open round circle (○)]. The activity was at the peak level when the PC concentration was 100 μ g. (D) Equilibrium CD spectra of 3β HSD2 in the presence of different concentrations of unilamellar lipid membranes (PC abbreviated as PC) from 200 to 240 nm. The black solid line [black square (■)] shows the CD spectrum of 3β HSD2 in the absence of lipid, whereas the red line 3β HSD2 [red closed circle (●)] is the spectrum in the presence of 50 μ g PC and blue line [blue closed triangle (▲)] is the spectrum with 250 μ g PC. The inset shows the enlarged view of the change in 3β HSD2 conformation at $\pi-\pi^*$ position in presence 250 μ g of PC. (E) Thermal unfolding (T_m) of 3β HSD2 in the presence and absence of lipid vesicles (100 μ g). Thermal denaturation was measured in a CD spectropolarimeter by monitoring the ellipticity at 222 nm. We precisely identified the T_m using the derivative $d\theta/dT$. The black line shows the measurement of 3β HSD2 stability based on T_m in the presence of PC, and the red line in the absence of PC. The inset shows the derivative $d\theta/dT$ with a peak at 42°C. Data presented are the mean $\pm$ S.E.M. from three independent experiments performed in triplicate. The bottom panels are western blots with the antibodies (Ab) against 3β HSD2 (42 kDa), VDAC2 (32 kDa), or Tom22 (22 kDa) showing that identical concentrations of mitochondria were used.

association. To understand the compartmental location and membrane integration of 3β HSD2 into the IMS, we synthesized 3β HSD2 in a CFS labeled with [35 S]methionine and imported into the isolated mitochondria from human adrenal NCI-H295R cells. We then characterized 3β HSD2's import proteolysis with PK in the presence and absence of Triton X-100. 3β HSD2 imported into the IMS should be protected from mild proteolysis by the OMM, but the addition of Triton X-100 allows the entry of PK through the OMM to the IMS to proteolyze 3β HSD2 (Hegde et al., 1998; Bose et al., 2002a). Figure 5A shows that the imported mitochondrial fraction was protected by the OMM from PK but was proteolyzed in the presence of Triton X-100. Western blotting with our human 3β HSD2 antibody showed identical amounts of mitochondria present in each reaction during the translocation experiments (Fig. 5A, bottom gel panel). To confirm that 3β HSD2 is indeed imported into the mitochondria, we separated the unimported fraction from the imported fraction (following centrifugation and washing with mitochondrial import buffer) and determined that most of the

fraction remained in the pellet. To confirm membrane association, we extracted the imported fraction with freshly prepared 100 mM Na_2CO_3 . The protein-protein interaction, but not the lipid-protein interaction, is disrupted in highly basic condition at or above pH 11.5 (Li and Shore, 1992). We found that 3β HSD2 was returned back in the supernatant under these conditions, suggesting that 3β HSD2 was not integrated into the mitochondrial membrane (Fig. 5B). To confirm that 3β HSD2 was imported into the IMS, we fused N-terminal 50 amino acids of Tim50 before the N terminus of the 3β HSD2 amino acid sequence, resulting in $\text{NH}_2\text{-Tim50-(1-50)-}3\beta\text{HSD2-(1-372)-COOH}$. Tim50 is an inner mitochondrial resident protein that is a part of the Tim23 mitochondrial translocase complex that helps in translocating mitochondrial preproteins (Geissler et al., 2002; Yamamoto et al., 2002; Pawlak et al., 2011). The results show that the cell-free synthesized Tim50- 3β HSD2 (54 kDa), when imported into the mitochondria, cleaves off a portion of the N-terminal Tim50 amino acids, leaving the entire 3β HSD2 sequence with

Fig. 4. Analysis of the conformational changes after the interaction of 3β HSD2 with the inhibitor steroids. Far-UV CD of 3β HSD2 protein is recorded between 195 and 250 nm after addition of androstenedione, cortisol, and cortisone at concentrations determined by their relative K_i values. (A) The far-UV CD spectra of solubilized, purified 3β HSD2 at 195–250 nm before and after addition of cortisone (10 μ M) and androstenedione (A'dione, 2 μ M). (B) The far-UV CD spectra of solubilized, purified 3β HSD2 at 195–250 nm after addition of cortisol (Cort, 100 μ M). (C) Similar far-UV CD spectra of purified 3β HSD2 in the presence of PC vesicles (PC) before and after addition of identical concentrations of cortisone and androstenedione as done in (A). (D) Far-UV CD spectra of purified 3β HSD2 in the presence of PC vesicles (PC) before and after addition of identical concentrations of cortisol as in (B). The CD spectra represent measurements from six repetitions for experiments that were performed in duplicate.

a partial Tim50 presequence (48 kDa) (Fig. 5C). The import reaction was exposed to PK with and without the addition of 0.5% Triton X-100. Only the imported 48-kDa protein remained in the absence of Triton X-100 because PK could not traverse the OMM, but both the external 54-kDa Tim50- 3β HSD2 and the imported 48-kDa protein are proteolyzed by PK when Triton X-100 allows PK to cross the OMM. This is a unique property of 3β HSD2, a large 42-kDa protein, because, unlike most mitochondrial proteins, 3β HSD2 is imported into the IMS without its own N-terminal sequence being cleaved. Only smaller Tim-imported proteins have been identified as being imported without cleavage of the protein's signal sequence (Koehler, 2004). Western blot with the VDAC2 antibody shows that mitochondrial fractionation

was accurate (Fig. 5, B and C). In summary, 3β HSD2 is imported into the IMS, because Tim50 can only direct proteins up to the IMS.

Discussion

We recently found that the production of DHEA and DHEAS in human adrenal NCI-H295R cells was stimulated by cortisol and cortisone (Topor et al., 2011). In the same study, cortisol (50 μ M) competitively inhibited human 3β HSD2 in transfected COS-7 cells (Topor et al., 2011). The assay in that study measured the 3β HSD2/isomerase sequence of reactions (androstenedione formation from DHEA), whereas the enzyme assay in the current study measured

Fig. 5. Mitochondrial association of 3β HSD2. (A) Import of a CFS [35 S] 3β HSD2 into the isolated mitochondria (Mito) from NCI-H295R cells determined the import into the IMS by incubation with PK and allowed the PK entry into the mitochondria incubating together with 0.5% Triton X-100 (Triton). The western blots in the bottom panel show that 3β HSD2 was present in equal amounts in each experiment. (B) CFS [35 S]-labeled 3β HSD2 was imported into the isolated mitochondria of NCI-H295R cells and then the imported pellet fraction (P) into the mitochondria was separated from the unimported supernatant (S) fraction by centrifugation. Similarly, a sodium carbonate fraction of the 3β HSD2 was extracted from the nonintegrated supernatant (S) fraction after incubation of Na_2CO_3 (pH 11.5) followed by centrifugation at 13,000g for 30 minutes at 4°C. The bottom panels are western blots with the indicated antibodies, showing that the 3β HSD2 (42 kDa) was present in the pellet fraction, and that equal amounts of mitochondria (VDAC2 marker at 32 kDa) were used for each experiment. (C) Confirmation of 3β HSD import. Tim50- 3β HSD2 [(1-50)Tim50- 3β HSD2] (54 kDa) was imported into the mitochondria, which incompletely cleaves off a portion of the N-terminal Tim50 amino acids, leaving the entire 3β HSD2 sequence with a partial Tim50 presequence (48 kDa). The import reaction was exposed to PK with and without addition of 0.5% Triton X-100. Only the imported 48-kDa protein remained in the absence of Triton X-100 because PK could not traverse the OMM, but both the external 54-kDa Tim50- 3β HSD2 and the imported 48-kDa protein are proteolyzed by PK when Triton X-100 allows PK to cross the OMM. The bottom two panels are western blots with the antibodies (Ab) against 3β HSD2 (42 kDa) and VDAC2 (32 kDa) showing that identical concentrations of mitochondria were used.

β 3HSD2 activity alone (NAD^+ to NADH at 340 nm). Determinations of enzyme kinetic constants and mode of inhibition are more accurate when the rate-limiting activity in the sequence is used (Segel, 1993). In our current study, we determined the kinetics of inhibition of β 3HSD2 by cortisol, cortisone, and androstenedione for both purified, solubilized β 3HSD2 (not bound to lipid vesicles) and the liposome-bound enzyme. We found that cortisol is a very low-affinity inhibitor of solubilized β 3HSD2 ($K_i = 542 \mu\text{M}$). To test whether membrane-bound, purified β 3HSD2 is inhibited with a kinetic profile similar to the enzyme expressed in COS-7 cells (Topor et al., 2011), we examined the inhibition kinetics of purified β 3HSD2 when bound to lipid vesicles.

Relative to purified, solubilized β 3HSD2, cortisol is a 2.5-fold better inhibitor of the liposome-bound enzyme, although even the liposome-bound K_i of $217 \mu\text{M}$ greatly exceeds the concentration of intra-adrenal cortisol (Dickerman et al., 1984; Nakamura et al., 2012). The inhibition mode of cortisol for both purified β 3HSD2 preparations is uncompetitive, unlike the competitive mode found in β 3HSD2-transfected COS-7 cells (Topor et al., 2011). Cortisone is a 1.6-fold more effective inhibitor of liposomal than solubilized enzyme, but is a much more effective inhibitor of β 3HSD2 than cortisol, having 10-fold and 6.4-fold lower K_i values than does cortisol for the solubilized and liposomal enzymes, respectively. Inhibition by cortisone is competitive for both enzyme preparations. Androstenedione competitively inhibits liposomal β 3HSD2 1.4-fold more effectively than solubilized β 3HSD2, and inhibits at much lower K_i values than cortisone or cortisol. The previous observations indicate that androstenedione may play a role in limiting the activity of β 3HSD2 during adrenarche, and that membrane association may enhance this inhibition. Our studies also show that androstenedione is a far better inhibitor of human β 3HSD2 than either cortisone or cortisol.

Enhancement of the inhibition of β 3HSD2 when the enzyme is bound to liposomes and the different modes of inhibition observed complement our studies of conformational analysis using CD spectra of steroid binding. There is a major shift in the CD spectrum at 208–220 nm of solubilized β 3HSD2 bound to cortisol. This cortisol-induced shift in the CD spectrum is also seen when β 3HSD2 is bound to liposomes, but cortisol induces a reduced spectral shift with liposome-bound β 3HSD2. Based on the shift in CD spectra at 208–220 nm, the binding of cortisone and androstenedione changes the conformation of liposome-bound β 3HSD2 less dramatically than cortisol, and does not affect the conformation of solubilized β 3HSD2 at all. Clearly, cortisol binds to β 3HSD2 very differently than does either cortisone or androstenedione.

As previously described (Rajapaksha et al., 2011, 2013a), hydrophilicity analysis has shown that the N-terminal β 3HSD2 sequence is more hydrophobic up to amino acid 120, and the amino acid sequence of the rest of the protein is weakly hydrophobic and does not form an amphiphilic helix, resulting in a favored association with the membrane. The association may be the result of an electrostatic interaction between the positively charged residues of the C terminus of the protein and the less polar cortisone and androstenedione. Modeling by fingerprinting in the presence of chaperones or lipid vesicles suggests that the initial N-terminal 93 amino acids become exposed to the surface, and thus have the

possibility of increased binding. The coil region of the C terminus is membrane-bound, leaving the N-terminal Rossmann fold region to associate with steroids. This might be the critical factor that allows β 3HSD2 to possess its two different steroidogenic activities, β 3HSD and steroid 5-4-ene isomerase.

Our mitochondrial import studies show that human β 3HSD2 is a membrane-associated enzyme in the IMS and is not an integral membrane protein in the inner or outer membrane of mitochondria. This suggests that the liposome-associated enzyme is a good model for our studies. Being at the IMS, where the mitochondrion is continuously moving, the β 3HSD2 protein must be very flexible. Thus, a transient electrostatic interaction could occur between the positively charged residues of β 3HSD2 and steroids, possibly forming a molten-disc conformation (Tamm et al., 2004). The chaperones at the inner mitochondrial membrane may help β 3HSD2 to become transiently stable and then go back to the active conformation. Alternatively, small structured domains of β 3HSD2 might interact with an as-yet unidentified receptor at the intermembrane space, and this receptor might be playing a significant role in facilitating the binding of specific steroids. In either event, our data suggest that the helices in the solubilized active β 3HSD2 structure could be preserved in the IMS (Bose et al., 1999), suggesting that the membrane-associated enzyme retains its flexible conformation. This must be a rapid process as steroid production is rapid to meet physiologic demands, suggesting that environmental factors play a significant role to maintain the conformation of β 3HSD2.

The trigger mechanisms for adrenarche during childhood remain unknown but must result from increased synthesis and/or decreased metabolism of DHEA. Steroid profiling during human adrenarche reveals an increase in 17,20-lyase activity as well as a decrease in β 3HSD2 activity (Rich et al., 1981; Lashansky et al., 1991; Palmert et al., 2001; Remer et al., 2005; Shi et al., 2009). 17,20-Lyase requires P450 oxidoreductase, cytochrome b_5 , and serine/threonine phosphorylation for maximal activity (Miller and Auchus, 2011). Adrenarche is associated with a major expansion of the zona reticularis, which expresses high levels of cytochrome b_5 , leading to selective stimulation of the 17,20-lyase activity of the bifunctional cytochrome P450 c17 to produce DHEA in preference to 17α -hydroxypregnenolone. The zona reticularis also expresses high levels of DHEA-sulfotransferase (SULT2A1) and low levels of both β 3HSD2 mRNA (Rege et al., 2014) and protein (Rainey et al., 2002; Rege and Rainey, 2012). Our inhibition data now suggest that the β 3HSD2 product steroid, androstenedione, may be a key player in limiting the metabolism of DHEA by β 3HSD2 in the zona fasciculata and reticularis during adrenarche. Using the liposome-bound β 3HSD2 kinetic values, androstenedione is a very potent competitive, product-feedback inhibitor ($K_i = 4.7 \mu\text{M}$) of the conversion of the DHEA substrate ($K_m = 13 \mu\text{M}$). A K_m/K_i ratio of 2.8 shows that androstenedione can decrease DHEA substrate conversion when just a small amount of product steroid has been formed by the enzyme, and explains a primary physiologic mechanism for the decrease in adrenal β 3HSD2 activity in adrenarche. This potent β 3HSD2 inhibition by androstenedione at physiologic intra-adrenal levels (Dickerman et al., 1984) is magnified by the limited expression of β 3HSD2 in the developing zona reticularis (Rege et al., 2014). Cortisone may also competitively

inhibit adrenal β HSD2, but much higher concentrations compared with androstenedione are required based on the measured K_i values. The liposome-bound β HSD2 K_i value for cortisone (34 μ M) is considerably higher than reported human adrenal levels (Dickerman et al., 1984; Nakamura et al., 2012). Based on the liposomal β HSD2 $K_i = 217 \mu$ M, cortisol appears to be a very low-affinity, uncompetitive inhibitor of β HSD2 that requires the enzyme to be bound to lipid membranes for inhibition even at high, nonphysiologic levels (Dickerman et al., 1984; Nakamura et al., 2012). Cortisol binds to the enzyme very differently than either cortisone or androstenedione based on CD spectral results and inhibition modes. Further studies are needed to fully assess a possible role for cortisol as an inhibitor of β HSD2 during human adrenarche.

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Authorship Contributions

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Wrote or contributed to the writing of the manuscript: Thomas, Majzoub, Bose.

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