Dehydroepiandrosterone and Its CYP7B1 Metabolite 7α-Hydroxydehydroepiandrosterone Regulates 11β-Hydroxysteroid Dehydrogenase 1 Directions in Rat Leydig Cells

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Background: The purpose of this study was to investigate cytochrome P450-7B1 (CYP7B1) in the human and rat testes to regulate 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) activity. We hypothesized that dehydroepiandrosterone (DHEA) and its product 7α-hydroxydehydroepiandrosterone (7αOHD) after catalysis of CYP7B1 played a critical role in driving the direction of 11β-HSD1, because 7αOHD is an alternative substrate for 11β-HSD1.

Methods: We examined the influence of DHEA and 7αOHD on 11β-HSD1 activities in both intact Leydig cells and microsomes using radioactive substrates and identified the location of CYP7B1 in Leydig cells using immunohistochemical staining, Western blot, and qPCR.

Results: We found that DHEA stimulated 11β-HSD1 oxidase activity in intact cells (EC50 = 0.97 ± 0.11 µM) and inhibited its reductase activity (IC50 = 1.04 ± 0.06 µM). In microsomes, DHEA was a competitive inhibitor of the reductase activity. The 11β-HSD1 oxidase activity in intact cells was inhibited by 7αOHD (IC50 = 1.18 ± 0.12 µM), and the reductase activity was enhanced (EC50 = 0.7 ± 0.04 µM). 7αOHD was a competitive inhibitor of 11β-HSD1 oxidase. CYP7B1 was present in rat Leydig cells, as shown by immunohistochemistry, Western blotting, and qPCR analysis.

Conclusion: Our results are consistent with a conclusion that DHEA in the circulation driving 11β-HSD1 toward an oxidase in Leydig cells mainly through inhibiting the reductase of the enzyme, while 7αOHD (CYP7B1 catalytic product of DHEA) drives the enzyme toward the opposite direction.

Keywords: steroids, dehydroepiandrosterone, 7α-hydroxydehydroepiandrosterone, leydig cells, CYP7B1
INTRODUCTION

11β-Hydroxysteroid dehydrogenases are a set of enzymes that catalyzes interconversion of active glucocorticoid (GC), cortisol (humans) or corticosterone (CORT, rodents), and inert GC, cortisone (humans) and 11-dehydrocorticosterone (DHC, rodents). There are two isoforms: type I (11β-HSD1) and type II (11β-HSD2) (1–4). 11β-HSD1 is an NADP+/NADPH-dependent bidirectional enzyme and is primarily present in liver and testis (5, 6). 11β-HSD2 is an unidirectional NAD+−dependent oxidase and is mainly present in kidney and colon (7).

The direction of 11β-HSD1 is influenced by NADP+/NADPH ratio in the smooth endoplasmic reticulum (SER). 11β-HSD1 is a membrane binding enzyme with cofactor-binding domain facing SER lumen (8–10). In liver cells, 11β-HSD1 co-localizes and shares NADP+/NADPH with hexose-6-phosphate dehydrogenase (H6PDH), which stays in SER lumen (11). H6PDH uses substrate, glucose 6-phosphate, which is transported into SER lumen by glucose 6-phosphate transporter (11). The membrane of SER is impermeable to NADP(H). The abundant supply of glucose 6-phosphate to H6PDH generates sufficient NADPH from NADP+. Increasing NADPH/NADP+ ratio and driving 11β-HSD1 toward as a reductase in liver cells, which generates NADP+ and 11β-hydroxysteroids.

As a major component of the pentose phosphate pathway, the cytosolic glucose-6-phosphate dehydrogenase, which accounts for 95% of NADPH amount generated in the whole cell, does not seem to affect the direction of 11β-HSD1 catalysis (12). This is supported by the fact that depletion of glucose-6-phosphate dehydrogenase by RNA silencing does not change the direction of 11β-HSD1 catalysis (12). In contrast, H6PDH, which accounts for only 5% of NADPH production, causes 11β-HSD1 to act as a reductase in liver cells, because these two enzymes and the co-factors are compartmentalized in SER lumen. Therefore, when H6PDH was knocked out, 11β-HSD1 in the liver became a primary oxidase (13).

However, 11β-HSD1 direction in other cell types may not be regulated by H6PDH (14). Unlike liver cells, 11β-HSD1 in rat adult Leydig cells (ALCs) are primarily an oxidase with comparable reductase activity (6). Although there is small amount of 11β-HSD2, which is only about 1/1000 of 11β-HSD1 in rat ALCs (15), its low abundance does not explain directionality of 11β-HSD in this cell type. Human Leydig cells also express 11β-HSD1 and 11β-HSD2 and Leydig cells can catalyze the interconversion of 11β-hydroxysteroids and 11keto-steroids (16). Other factors may also drive 11β-HSD1 direction in rat and human ALCs. These factors may include steroids from adrenals or steroids generated locally. In this regard, 11β-HSD1 oxidase activity was significantly reduced in ALCs isolated from adrenalectomized rats when compared to that in cells in adrenal-intact controls (17). Therefore, adrenal-secreted steroids might be involved in maintaining the direction of 11β-HSD1 in rat ALCs. Dehydroepiandrosterone (DHEA) is a steroid mainly synthesized by human adrenal glands and testes as a precursor for cortisol and testosterone, and is one of the most abundant steroids in human blood. DHEA can be considered a candidate regulator of 11β-HSD1 direction because of high circulating levels and structural resemblance to the substrate of 11β-HSD1.

DHEA is also the substrate for SER cytochrome P450-dependent enzyme CYP17B1, and which is expressed in various tissues, including brain, prostate, and testis (18, 19). CYP17B1 catalyzes the conversion of DHEA into 7α-hydroxydehydroepiandrosterone (7αOHD) and generates NADP+. Therefore, the metabolic coupling with CYP17B1 is also possible whereby NADP+ drives 11β-HSD1 oxidase activity. 7αOHD may be a regulator because it is a substrate of 11β-HSD1. The epimerase activity of 11β-HSD1 converts 7αOHD into 7β-dehydroepiandrosterone (7βOHD) (20–22). However, human, pig, and rat 11β-HSD1 also catalyze 7αOHD using NADP+ as cofactor into 7keto-DHEA (7KD) and NADPH (23). We hypothesize that DHEA in the circulation may affect 11β-HSD1 direction in three ways: by direct stimulation of the enzyme, or through metabolic coupling with CYP17B1, or by affecting the enzyme via the metabolite, 7αOHD. The goal of the present study was to investigate each of these possibilities.

MATERIALS AND METHODS

Chemicals and Animals

CORT, DHEA, and 7αOHD were purchased from Steraloids (Wilton, NH). [1,2-3H] Corticosterone (3H-CORT), specific activity 40 Ci/mmol, was purchased from Dupont-New England Nuclear (Boston, MA). [1,2-3H]11-dehydrocorticosterone (3H-DHC) was prepared from labeled 3H-CORT as described earlier (24).

Animals

Male Sprague-Dawley rats (from 2 to 90 days old) were purchased from Shanghai Laboratory Animal Co. Ltd. (Shanghai, China). Rats were sacrificed at postnatal days 2, 7, 14, 21, 28, 35, and 90 by asphyxiation with CO2. One testis per rat was removed and frozen in liquid nitrogen for RNA extraction. The other testis was punched 5 holes using a needle and was fixed by Bouin’s solution. The animal protocol was approved by the Institutional Animal Care and Use Committee of the Wenzhou Medical University.

Cell Isolation

Testes were processed for purification of Leydig cells. The progenitor (PLC), immature (ILC), and adult Leydig cells (ALC) were purified from rat testes at 21, 35, and 90 days postpartum, as described previously (25, 26). In brief, forty 21-day-old (for PLCs), eighteen 35-day-old (for ILCs), and six 90-day-old (for ALCs) rats were sacrificed, followed by removal and decapsulation of testes. Then, the testes were digested with collagenase and cells were separated by Percoll density gradient centrifugation. Purities of Leydig cell fractions were evaluated by histochemical staining of 3β-hydroxysteroid dehydrogenase (3β-HSD), with 0.4 mM etiocholanolone as an enzyme substrate (27). At least six isolations per cell types were performed.

Preparation of Microsomal Protein

The microsomes from ALCs were prepared as described previously (6). In brief, ALCs were homogenized in cold...
0.01M PBS, pH 7.2, containing 0.25M sucrose. Subsequently, the homogenates were centrifuged at 700 × g for 30 min and the supernatants were transferred to other tubes followed by centrifugation at 10,000 × g for 30 min. The resultant supernatants were centrifuged twice at 105,000 × g for 1 h. The pellets were resuspended and the protein contents were determined by Bio-Rad Protein Assay Kit (cat# 500–0006, Bio-Rad, Hercules, CA). Microsomes were used to assay 11β-HSD1 oxidoreductase activities.

**Primer Selection**

All primers in this study were chosen using a sequence analysis software package (Primer 3, Whitehead Institute for Biomedical Research, Cambridge, MA) following guidelines for internal stability. Forward and reverse primers were in different exons to minimize the effects of possible DNA contamination. The primers for Cyp7b1 were: 5'-GAAGTCCTGCGTGACGAAAT-3' (forward); 5'-CCTCAGAACCTCAAGAATAGCG-3' (reverse); and the size of PCR product is 138 bp. For the internal standard, primers to ribosomal protein S16 (Rps16) were used, as described previously (28).

**Real-Time PCR**

PCR was carried out in a 25 µl volume using a 96-well plate format using the SYBR Green PCR Core Reagents purchased from Applied Biosystems (Foster City, CA). Primer titration was performed and the concentration of 300 nM was selected. Fluorescence was detected in real-time on an ABI 5700 system (PE Applied Biosystems). The mRNA levels were measured by a standard curve method and the levels were normalized to Rps16, the internal control. Each sample was run in duplicate, in parallel with no template controls.

**Immunofluorescent Staining**

Immunofluorescent staining of CYP7B1 was performed on isolated ALCs using cells grown on 12-well microscope cover glasses. Cells were fixed with 4% formaldehyde, washed with PBS, and permeabilized with 0.1% Triton X100 in PBS plus 10% normal serum. Non specific binding was blocked by incubation with 10% normal serum before addition of the primary antibody. Cells were incubated with goat polyclonal CYP7B1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. Cells were then incubated with Alexa488-conjugated second antibody for 1 h. The cells were counterstained with DAPI and mounted onto glass microscope slides, and cover-slipped. The slides were examined under a Nikon fluorescence microscope with a filter suitable for detecting the fluorescence of green color.

**Immunohistochemical Staining**

Testes were dehydrated in ethanol and xylene and embedded in paraffin for immunological analysis. 6 µm-thick transverse sections were prepared and mounted on glass slides. Avidin–biotin immunostaining was performed using a Vector kit (Burlingame, CA) according to the manufacturer’s instructions. Antigen retrieval was carried out by boiling for 10 min in 10 mM (pH 6.0) citrate buffer, and endogenous peroxidase was blocked with 0.5% H₂O₂ in methanol for 30 min. The sections were then incubated with goat polyclonal CYP7B1 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. The antibody-antigen complexes were visualized with diaminobenzidine alone, resulting in brown cytoplasmic staining in positively labeled cells. The sections were counterstained with Mayer hematoxylin, dehydrated in graded concentrations of alcohol, and cover-slipped with resin (Permunt, SP15–100; Fisher Scientific).

**Western Blotting**

Western blotting was performed as previously described (29). Briefly, rat PLC, ILC, and ALC homogenates (30 µg protein each sample) were electrophoresed on 10% polyacrylamide gel containing sodium dodecyl sulfate. After electrophoresis, proteins were transferred onto a nitrocellulose membrane, and the membrane was incubated with 5% non-fat milk for 1 h to block the non-specific binding. Then, the membrane was incubated with primary antibodies against the following antigens: CYP7B1 (Santa Cruz Biotechnology) and β-actin (ACTB, Cell Signaling Technology, Danvers, MA), which serves the internal control. The membrane was washed and incubated with a 1:5,000 dilution of secondary antibody conjugated to horseradish peroxidase. Immunoreactive bands were visualized by an ECL kit (Amersham, Arlington Heights, IL). The intensity of the protein was quantified using Image J software. The CYP7B1 was adjusted to ACTB.

**11β-HSD1 Oxidase and Reductase Assay in Intact ALCs**

11β-HSD1 oxidase and reductase in intact ALCs were performed as previously described (15). 11β-HSD1 oxidase activity assay tubes contained 25 nM CORT spiked with 60,000 dpm [3H]-CORT (a concentration within the range of physiological levels of CORT). 11β-HSD1 reductase activity assay tubes contained 25 nM DHC spiked with 60,000 dpm [3H]-DHC. 2.5 × 10⁴ intact ALCs were added to start the reaction and the reaction was maintained in 34°C and 75 rpm shaking water bath for 30 min. The reactions were stopped by adding 2 ml ice-cold ether. The steroids were extracted, and the organic layer was dried under nitrogen. The steroids were separated chromatographically on thin layer plates in chloroform and methanol (90:10, v/v), and the radioactivity was measured using a scanning radiometer (System AR2000, Bioscan Inc., Washington, DC). The percentage conversion of CORT to DHC and DHC to CORT was calculated by dividing the radioactive counts identified as DHC (or CORT, respectively) by the total counts associated with CORT plus DHC.

**11β-HSD1 Oxidase and Reductase Assay in Microsomes**

11β-HSD1 oxidase and reductase in microsomes were performed as previously described (15). For measurements in microsomal preparations, microsomes were incubated with 25 nM [3H]-CORT and 0.2 mM NADP⁺ for oxidase or 25 nM [3H]-DHC and 0.2 mM NADPH for reductase activity in presence of different concentrations of DHEA or 7αOHD. For testing mode of action, microsomes were incubated with 2 nM to 10 µM [3H]-CORT
and 0.2 mM NADP⁺ for oxidase or 2 nM to 10 µM [³H]-DHC and 0.2 mM NADPH for reductase activity. 1.5 µg of ALC microsomes were added to initiate the reaction. The reactions were stopped by adding 2 ml ice-cold ether. The steroids were extracted, and the organic layer was dried under nitrogen. The steroids were separated chromatographically on thin layer plates in chloroform and methanol (90:10, v/v), and the radioactivity was measured using a scanning radiometer (System AR2000, Bioscan Inc., Washington, DC). The percentage conversion of CORT to DHC and DHC to CORT was calculated by dividing the radioactive counts identified as DHC (or CORT, respectively) by the total counts associated with CORT plus DHC.

Statistics
Experiments were repeated three to four times. Data were subjected to analysis by one-way ANOVA followed by ad hoc DUNCAN multiple comparisons testing to identify significant differences between groups when three and more groups were calculated, or by the student t-test when two groups were calculated. All data are expressed as means SEM. Differences were regarded as significant at *P < 0.05 or **P < 0.01 or ***P < 0.001.

RESULTS
DHEA Decreases 11β-HSD1 Reductase Activity in Intact ALCs
11β-HSD1 reductase converts DHC into CORT. We measured 11β-HSD1 reductase activity in intact ALCs in presence of DHEA (10 nM–10 µM). DHEA inhibited 11β-HSD1 reductase activity with the lowest observable effective level (LOEL) of 100 nM and it lowered 11β-HSD1 reductase activity to 12% of control at 10 µM (Figure 1A). The IC₅₀ value of DHEA of inhibiting 11β-HSD1 reductase activity was 1.04 µM (Table 1). This indicates that DHEA is a potent inhibitor of 11β-HSD1 reductase in intact ALCs.

DHEA Increases 11β-HSD1 Oxidase Activity in Intact ALCs
11β-HSD1 oxidase converts CORT into DHC. We measured 11β-HSD1 oxidase activity in intact ALCs in presence of DHEA (10 nM–10 µM). DHEA stimulated 11β-HSD1 oxidase activity with LOEL of 100 nM (Figure 1B). DHEA increased 11β-HSD1 oxidase activity to 300% of control at 10 µM. The EC₅₀ value of DHEA of stimulating 11β-HSD1 oxidase activity was 0.97 µM (Table 1). We calculated 11β-HSD1 oxidase/reductase ratio (Figure 1C) and found that 11β-HSD1 oxidase/reductase ratio in the control was 1.6-fold conforming to the previous report (17). DHEA increased this ratio by 26-folds at 10 µM, indicating that DHEA makes 11β-HSD1 more oxidative.

DHEA Inhibits 11β-HSD1 Reductase Activity in ALC Microsomes
In order to separate the potential contribution of the pentose phosphate pathway that is located in the cytosol, 11β-HSD1 reductase activity was measured in ALC microsomes in presence of DHEA (10 nM–10 µM). DHEA decreased ALC microsomal 11β-HSD1 reductase activity with LOEL of 100 nM and it lowered 11β-HSD1 reductase activity to 25% of control at 10 µM (Figure 2A). The IC₅₀ value of DHEA of inhibiting 11β-HSD1 reductase activity in ALC microsomes was 1.29 µM (Table 1). This is similar to what was found in intact ALCs.

DHEA Does Not Affect 11β-HSD1 Oxidase Activity in ALC Microsomes
11β-HSD1 oxidase activity was measured in ALC microsomes in presence of DHEA (10 nM–10 µM). DEHA did not affect microsomal 11β-HSD1 oxidase activity. Actually, DHEA also inhibited 11β-HSD1 oxidase activity at 10 µM (Figure 2B). Although DHEA did not affect 11β-HSD1 oxidase, 11β-HSD1 oxidase/reductase ratios in ALC microsomes were still significantly higher than control at 1 and 10 µM DHEA (Figure 2C). These data indicate that the stimulation of 11β-HSD1 oxidase by DHEA requires intact ALCs.

CYP7b1 Is Enriched in Rat ALCs
We determined that the timing of Cyp7b1 expression in Leydig cells was developmentally relevant. Cyp7b1 mRNA levels detected by qPCR in templates prepared from rat testis on postnatal days 2–90 did not vary significantly (Figure 3). When Leydig cells were enriched in developmental stages (PLCs at 21 days, ILCs at 35 days, and ALCs at 90 days postpartum), Cyp7b1 mRNA was 3-folds more abundant in ILCs and 50-folds more abundant in ALCs than that in PLCs (Figure 3). Cyp7b1 mRNA levels were enriched by 33-folds in ALCs when compared to age-matched testes. This suggests that Cyp7b1 is primarily expressed in ALCs.

CYP7B1 Protein Is Present in Rat ALCs
Previous studies have demonstrated that CYP7B1 (encoded by Cyp7b1) catalyzes DHEA into 7αOHD and that the epimerase activity of 11β-HSD1 converts 7αOHD into 7βOHD (20–22). Besides human, pig and rat 11β-HSD1 can catalyze 7αOHD using NADP+ into 7KD to generate NADPH (23). In order to investigate a potential metabolic coupling between CYP7B1 and 11β-HSD1, we first sought to show whether CYP7B1 was present in rat ALCs. CYP7B1 was detected in ALCs by fluorescent immunohistochemistry and CYP7B1 was present in the cytoplasmic membrane (Figures 4A–E) when compared to the negative control (Figure 4F). Western blotting also showed the highest expression of CYP7B1 in rat ALCs (Figure 4G). This suggests that CYP7B1 is primarily expressed in ALCs.

7αOHD Increases 11β-HSD1 Reductase Activity in Intact ALCs
CYP7B1 converts DHEA into 7αOHD. 11β-HSD1 reductase activity was measured in intact ALCs in presence of 7αOHD (10 nM–10 µM). 7αOHD increased 11β-HSD1 reductase activity with LOEL of 100 nM (Figure 5A). 7αOHD increased 11β-HSD1 reductase activity to 200% of control at 1 and 10 µM (Figure 5A). The EC₅₀ value of 7αOHD of increasing 11β-HSD1 reductase activity was 0.7 µM (Table 1). This suggests that 7αOHD does the opposite action of DHEA on 11β-HSD1 reductase activity in intact ALCs.
Regulation of 11β-HSD1 by CYP7B1

**FIGURE 1** | Effects of dehydroepiandrosterone (DHEA) on 11β-HSD1 oxidase and reductase activities in intact adult Leydig cells (ALCs). 2.5 × 10⁷ ALCs were cultured with 25 nM DHC (reductase, A) or CORT (oxidase, B) for 0.5 h. (C) is 11β-HSD1 oxidase/reductase ratio. Mean ± SEM (n = 4). *, **, and *** designate significant differences when compared to control (first bar in each panel) at P < 0.05, 0.01, and 0.001, respectively.

**TABLE 1** | EC₅₀ or IC₅₀ for the regulation of 11β-HSD1 oxidase and reductase activities in intact Leydig cells and microsome.

| Chemicals | EC₅₀/IC₅₀ (× 10⁻⁶ M) | Intact Leydig cells | Microsome |
|-----------|-----------------------|---------------------|-----------|
|           |                       | Oxidase             | Reductase | Oxidase | Reductase |
| DHEA      | EC₅₀/IC₅₀             | 0.97 ± 0.10         | 1.04 ± 0.06 | ND      | 1.29 ± 0.89 |
| 7αOHD     | EC₅₀/IC₅₀             | 1.18 ± 0.12         | 0.7 ± 0.04  | 0.93 ± 0.14 | 0.2 ± 0.01 |

ND, not detected; Mean ± SEM (n = 4).

**FIGURE 2** | Effects of dehydroepiandrosterone (DHEA) on 11β-HSD1 oxidase and reductase activities in adult Leydig cell (ALC) microsomes. 1.5 µg ALC microsome were cultured with 25 nM DHC plus 0.2 mM NADPH (reductase, A) or 25 nM CORT plus 0.2 mM NADP⁺ (oxidase, B) for 0.5 h. (C) is 11β-HSD1 oxidase/reductase ratio. Mean ± SEM (n = 4). *, **, and *** designate significant differences when compared to control (first bar in each panel) at P < 0.05, 0.01, and 0.001, respectively.

7αOHD Decreases 11β-HSD1 Oxidase Activity in Intact ALCs

11β-HSD1 oxidase activity was measured in intact ALCs in presence of 7αOHD (10 nM–10 µM). 7αOHD decreased 11β-HSD1 oxidase activity with LOEL of 100 nM (Figure 5B). 7αOHD decreased 11β-HSD1 oxidase activity to 60% of control at 1 µM and 25% of control at 10 µM (Figure 5B). The IC₅₀ value of 7αOHD of inhibiting oxidase activity was 1.17 µM (Table 1). We calculated 11β-HSD1 oxidase/reductase ratio (Figure 5C). 7αOHD decreased 11β-HSD1 oxidase/reductase ratio to 50% of control at 1 µM and to 20% of control at 10 µM. This indicates that 7αOHD makes 11β-HSD1 more reductive.

7αOHD Increases 11β-HSD1 Reductase Activity in ALC Microsomes

11β-HSD1 reductase activity was measured in ALC microsomes in presence of 7αOHD (10 nM–10 µM). 7αOHD increased 11β-HSD1 reductase activity with LOEL of 100 nM. However, at the highest concentration (100 nM), 7αOHD did not affect
11β-HSD1 reductase activity (Figure 6A). The EC_{50} value of 7αOHD of increasing 11β-HSD1 reductase activity was 200 nM (Table 1).

7αOHD Inhibits 11β-HSD1 Oxidase Activity in ALC Microsomes

11β-HSD1 oxidase activity was measured in ALC microsomes in presence of 7αOHD (10 nM–10 μM). 7αOHD decreased 11β-HSD1 oxidase activity with LOEL of 100 nM (Figure 6B). 7αOHD decreased 11β-HSD1 oxidase activity to 60% of control at 1 μM and to 25% of control at 10 μM (Figure 6B). The IC_{50} value of 7αOHD for inhibiting oxidase activity was 0.93 μM (Table 1). 7αOHD significantly decreased 11β-HSD1 oxidase/reductase ratio in ALC microsomes at 0.1–10 μM (Figure 6C). The action of 7αOHD in ALC microsomes was similar to that in intact ALCs, indicating that 7αOHD acts on 11β-HSD1 within SER.

DHEA Is a Competitive Inhibitor of 11β-HSD1 Reductase in ALC Microsomes

We measured the rates of reductase reactions in ALC microsomes in presence of different DHEA concentrations (0, 1, and 10 μM) in order to evaluate the mode of action. DEHA was a competitive inhibitor of 11β-HSD1 reductase (Figure 7A).

7αOHD Is a Competitive Inhibitor of 11β-HSD1 Oxidase in ALC Microsomes

We measured the rates of oxidase reactions in ALC microsomes in presence of different 7αOHD concentrations (0, 1, and 10 μM) in order to evaluate the mode of action. 7αOHD was a competitive inhibitor of 11β-HSD1 oxidase (Figure 7B).

DISCUSSION

The present study demonstrated that DHEA stimulated 11β-HSD1 oxidase activity and inhibited 11β-HSD1 reductase activity in intact ALCs. The metabolite of DHEA by CYP7B1, 7αOHD, inhibited 11β-HSD1 oxidase activity and stimulated 11β-HSD1 reductase activity in intact ALCs and in ALC microsomes.

CYP7B1 and 11β-HSD1 coexist in rat ALCs. The detectable Cyp7b1 mRNA and histochemical staining and Western blotting analysis of CYP7B1 indicate that ALCs are the major, if not exclusive, sites of Cyp7b1 expression. We showed that Cyp7b1 mRNA levels in purified ALCs were enriched by 33-folds when compared to age-matched testis samples (Figure 3). Developmental increases in Cyp7b1 mRNA levels in purified ALCs were not distinct in the entire testis, but were significant in the purified Leydig cells (Figure 3). Compared to PLCs, Cyp7b1 mRNA levels were at least 3-folds higher in ILCs and at least 50-folds higher in ALCs (Figure 3). We infer that CYP7B1 is associated with the development and maturation of Leydig cells. Whether LH affects Cyp17b1 expression is unclear and requires future study. Since Cyp7b1 is developmentally...
increased in Leydig cells (Figure 3) and LH is the major factor to regulate Leydig cell development postnatally (30), we speculate that LH can affect Cyp17b1 expression in rat Leydig cells.

We investigated whether CYP7B1 was coupled with 11β-HSD1. CYP7B1 catalyzes the conversion of DHEA into 7αOHD using NADPH as cofactor (18). The product of NADP⁺ from this reaction might create an environment that accelerates 11β-HSD1
oxidase activity. We tested the hypothesis by adding DHEA to intact ALCs, and found that 11β-HSD1 oxidase activity was significantly increased (Figure 1B). Therefore, it was likely that addition of DHEA drove the formation of NADP⁺. However, the experiment was performed in intact cells. We could not dissect the possible sources of NADP⁺. Therefore, we repeated the experiment by adding DHEA to ALC microsomes, and found that 11β-HSD1 oxidase activity was nearly unchanged (Figure 2B). These results indicate that the stimulation of 11β-HSD1 oxidase activity by DHEA requires cytosolic NADP⁺ pool. Indeed, DHEA has been reported to inhibit glucose-6-phosphate dehydrogenase and increase the cytosolic pool of NADP⁺, which would also drive 11β-HSD1 oxidase activity (31). The present study also showed DHEA was a potent competitive inhibitor of 11β-HSD1 reductase, but not of 11β-HSD1 oxidase. We showed a concentration dependent increase in the ratio of 11β-HSD1 oxidase to reductase (Figure 1C).

7αOHD, the product of DHEA by CYP7B1, is also a substrate for human 11β-HSD1. The conversion of 7αOHD to 7KD by 11β-HSD1 is bidirectional (23). Although human 11β-HSD1 was shown to have epimerase activity, which converts 7αOHD into 7βOHD (32), human, pig and rat 11β-HSD1 can catalyze 7αOHD into KD to generate NADPH (23). 7αOHD was proposed as a native competitor for the 11β-HSD1-mediated activation of cortisone into cortisol (20). However, in the present study, 7αOHD inhibited 11β-HSD1 oxidase and increased 11β-HSD1 reductase, which would have the result of amplifying glucocorticoid actions by converting more cortisone into cortisol (or 11DHC to CORT in rats). 7αOHD was a competitive inhibitor of 11β-HSD1 oxidase (Figure 7B). This is consistent with the finding that 7αOHD shares the active site for cortisone in human 11β-HSD1 (32). The exact mechanism of this inhibition will require further investigation.

The actual physiological impact of 7αOHD is not likely to result in amplification of GC actions. The circulating levels of DHEA sulfate (DHEAS) or DHEAS plus DHEA are much higher than those of cortisol (33–35), these in turn are about three orders of magnitude larger (in humans) than levels of 7αOHD (36, 37). Therefore, among the circulating steroids, DHEA might have more influence than 7αOHD on 11β-HSD1 activities in ALCs, and the oxidase direction will be favored. Although 7αOHD can be generated locally from DHEA by CYP7B1, the relative abundance of CYP7B1 versus 11β-HSD1 will be a factor. In the present study, we found that Cyp7b1 expression was much lower (300 copy/pg RNA in rat ALCs) compared to 11β-HSD1 mRNA levels in ALCs (500,000 copy/pg RNA) (15). If the mRNA levels are indicative of the actual enzyme levels, it is unlikely that enough 7αOHD will be produced to change the direction of 11β-HSD1.

In summary, DHEA directly inhibits 11β-HSD1 reductase in the intact ALCs, thus default 11β-HSD1 is enhanced to drive 11β-HSD1 being more oxidative (Figure 8A). CYP7B1 is present in Leydig cells and can catalyze the formation of 7αOHD in ALCs. 7αOHD is the substrate of 11β-HSD1 oxidase, thus competitively inhibiting 11β-HSD1 oxidase to drive 11β-HSD1 being more reductive (Figure 8B). The study is relevant and contribution to endocrinological regulation of testicular GC contents by adrenal steroids. However, the expression of Cyp7b1 is about 1/1700 of Hsd11b1 and therefore the action of CYP7B1 may be limited.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Wenzhou Medical University. Written informed consent was obtained...
from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

JS and R-SG conceptualized the study design and analyze the data. QZ, YD, XL, CN, and TH performed the experiments and collected the data. R-SG wrote the manuscript and has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis, and all authors reviewed the manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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