The Adrenal Lipid Droplet is a New Site for Steroid Hormone Metabolism

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Abstract

Steroid hormones play essential roles for living organisms. It has been long and well established that the endoplasmic reticulum (ER) and mitochondria are essential sites for steroid hormone biosynthesis because several steroidogenic enzymes are located in these organelles. The adrenal gland lipid droplet (LD) proteomes from human, macaque monkey, and rodent were analyzed, revealing that steroidogenic enzymes are also present in abundance on LDs. The enzymes found include 3β-hydroxysteroid dehydrogenase (HSD3B), and estradiol 17β-dehydrogenase 11 (HSD17B11). Analyses by Western blot and subcellular localization consistently demonstrated that HSD3B2 was localized on LDs. Furthermore, *in vitro* experiments confirmed that the isolated LDs from HeLa cell stably expressing HSD3B2 or from rat adrenal glands had the capacity to convert pregnenolone to progesterone.

Collectively, these data suggest that LDs may be important sites of steroid hormone metabolism. These findings may bring novel insights into the biosynthesis and metabolism of steroid hormones and the development of treatments for adrenal disorders.

Keywords: adrenal; lipid droplets (LDs); steroid hormone metabolism; 3β-hydroxysteroid dehydrogenase 2 (HSD3B2).

Statement of significance:

The adrenal lipid droplets (LDs) mainly store cholesterol esters that serve as precursors for steroid hormone biosynthesis. In this report, we isolated adrenal lipid droplets from human, monkey, rat, and swine, and found that many enzymes involved in steroid hormone metabolism, such as 3β-hydroxysteroid dehydrogenase (HSD3B) and estradiol 17β-dehydrogenase 11 (HSD17B11), were enriched in LD proteomes. Biochemical and microscopy methods confirmed the localization of HSD3B2 on LDs. Furthermore, an *in vitro* assay of enzymatic activity demonstrated that the isolated LDs with HSD3B2 were able to converting pregnenolone to progesterone. Collectively, this work suggests that LDs may be important sites for steroid hormone metabolism, in addition to the well-known mitochondria

and endoplasmic reticulum. These findings may bring novel insights into the biosynthesis and metabolism of steroid hormones, as well as treatments for adrenal disorders.

1. Introduction

Steroid hormones such as cortisol, aldosterone, estrogens, progesterone, and androgens are indispensable for mammalian life, playing essential roles in metabolism, inflammation, immune functions, and sex characteristic development. Cholesterol is the common precursor for the biosynthesis of all steroid hormones. In the steroidogenic cells of the adrenals, gonads and placenta, cholesterol originating from lipid droplets (LDs), *de novo* synthesis, or extracellular lipoproteins [1-3], is transported into mitochondria via steroidogenic acute regulatory protein (StAR) [1, 4]. This 37 kDa protein, containing a typical mitochondrial leader sequence, transfers cholesterol across both the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) [5].

The conversion of cholesterol to various steroid hormones is catalyzed by several members of the cytochrome P450 superfamily [3]. Synthesis is initiated by cholesterol side-chain cleavage enzyme CYP11A1 (P450scc) which converts cholesterol to pregnenolone, which is further catalyzed to progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD, or HSD3B). Progesterone is subsequently converted to cortisol, corticosterone or aldosterone

through a sequence of reactions catalyzed by distinct P450 enzymes, including P450c17 (17α-hydroxylase and 17, 20 lyase, CYP17A1), P450c21 (21-hydroxylase, CYP21A), P450c11β (11β-hydroxylase, CYP11B1), and P450c11AS (CYP11B2) [3, 6]. The enzyme P450scc (CYP11A1) and its two electron-transfer partners, ferredoxin reductase and ferredoxin, are rate-limiting enzymes of steroidogenesis that determine net steroidogenic capacity.

It has been long and well established that both mitochondria and the endoplasmic reticulum (ER) are essential sites for steroid hormone biosynthesis [3]. Steroidogenic enzymes including CYP11A1 (P450scc), CYP11B1 (P450c11β) and CYP11B2 (P450c11AS) reside in the mitochondria, and HSD3B resides in both the mitochondria and endoplasmic reticulum (ER) [3, 6]. However, it is completely unknown how intermediate products like pregnenolone shuttle between mitochondria and the ER where CYP17A1 (P450c17) and CYP21A (P450c21) are located. Meanwhile, although StAR has been widely thought to mediate the flow of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) for CYP11A1 (P450scc) catalysis, some steroidogenesis still occurs in the absence of StAR [7, 8]. Collectively, these observations suggest that steroidogenesis may also occur in and involve another intracellular organelle besides the mitochondria and ER.

Lipid droplets (LDs) are now widely recognized for their active participation in cellular lipid metabolism. These organelles consist of a core of neutral lipids, including triglycerides (TG) and cholesterol esters (CE), surrounded by a monolayer composed of phospholipids and proteins [9, 10]. LDs are near ubiquitous, found in cells of various organisms from bacteria to mammals [11]. Numerous studies using molecular biology, biochemistry and proteomics have illuminated the protein composition of lipid droplets, including PLIN1/perilipin [12], PLIN2/adipocyte differentiation-related protein (ADRP) [13], PLIN3/tail-interacting protein of 47 kDa (TIP47) [14], adipose triglyceride lipase (ATGL), and Rab proteins [15]. Recent research has provided clear evidence that LDs play an active role in lipid metabolism, including lipid synthesis, lipolysis, lipid trafficking, and interactions with other organelles in response to changes in both intracellular and extracellular energy states [10, 16].

The neutral lipid composition of LDs in the adrenal cortex and gonads is dominated by cholesterol esters which serve as precursors for steroid hormone biosynthesis [1, 10, 17, 18]. Improved LD isolation methods coupled with proteomics have provided advantages for the investigation of LD function and have uncovered an apparent role of LDs in lipid homeostasis [18, 19]. Our and other lab's recent reports revealed that hydroxysteroid dehydrogenase (HSD) family was found on LDs by LD proteomic analysis across organisms from oleaginous bacteria RHA1 (HSD protein, ro01416), *C. elegans* (DHS-3), to mammalian cells (HSD17B11 and HSD17B13) [15, 20, 21]. Normally, HSD17B11 localizes in the

endoplasmic reticulum (ER), but it is redistributed between both ER and LDs when mice are fed a diet containing a peroxisomal proliferator-activated receptor-alpha agonist [22]. We discovered that another member of the 17β-hydroxysteroid dehydrogenase family, HSD17B13, a protein involved in the development of non-alcoholic fatty liver disease (NAFLD), is also localized on LDs [21].

A recently published study investigating rat granulosa cells treated with high density lipoproteins (HDL) or fatty acids (FAs) found differences between the proteomes of LDs containing primarily cholesterol esters (CEs) and those containing triacylglycerides (TAGs) [18]. However, the significance of this result is not clear since the compounds used to induce the LDs also act as signaling molecules that remodel cells [23]. Thus, we decided to study the nature of LDs that mainly store CEs by analysis of adrenal LDs. We hypothesized that steroidogenic enzymes may be also localized on LDs in adrenal cells and that these LDs are sites for synthesis of certain steroid hormones.

We isolated LDs from adrenal glands of rodent, macaque monkey, and human. Proteomic analysis of these adrenal LDs demonstrated that steroidogenic enzymes are consistently highly abundant in the LD fraction. Furthermore, *in vitro* biochemical experiments revealed that the isolated adrenal LDs were able to convert pregnenolone to progesterone, in a HSD3B dependent manner. These results suggest that LD of the adrenal gland is a site of steroid hormone metabolism.

2. Experimental Section

2.1. Human samples

The collection of clinical information and adrenal gland samples from two patients was approved by the Ethical Committee on Human Research of the General Hospital of the Air Force and was conducted with patient consent. The tissue donors had been admitted to the General Hospital of Air Force for the diagnosis and treatment of adrenal gland and/or kidney diseases. The female patient was diagnosed with cancer of the left kidney, and the male patient had cancer in the right adrenal gland. Their adrenal glands were removed pursuant to their therapy and the organ samples were used for LD purification.

2.2. Animal studies

Adrenal glands were obtained from rhesus macaques (*Macaca mulatta*) that were raised at the Kunming Institute of Zoology, Chinese Academy of Sciences. Rat adrenal glands were obtained from ten-week-old Sprague Dawley rats (Vital River Laboratories). Fresh adrenal glands of castrated swine were obtained from the slaughterhouse and transported to the laboratory as soon as possible. Brown adipose tissue (BAT) was from eight-week-old mice

(Vital River Laboratories). All of the procedures were approved by the Animal Care and Use Committee of the Institute of Biophysics, Chinese Academy of Sciences [Approval number: SYXK (SPF 2011-0029)].

2.3. Lipid droplet (LD) isolation and LipidTox staining

LDs were purified from adrenal glands and liver using a previously described method [24], to isolate LDs from BAT, but with a modified centrifugal force of 10,000 *g*. The total membranes formed a pellet following the 25,000 *g* centrifugation. They were resuspended in Buffer B (20 mM HEPES (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$), and were washed with Buffer B for two times. The cytosol was fractionated by centrifugation at 135,000 *g* (OptimaTM Ultracentrifuge TLA 100.3) for 20 min. LDs were purified from Hela cells stably expressing HSD3B2, PLIN2 or GFP as previously described. Purified LDs were resuspended in Buffer B and were stained with LipidTox at a ratio of 1:100 (v/v) (LipidTox:LDs) on ice for 20 min. The preparation was then visualized with an inverted microscope (Zeiss AxioImager M2 Imaging System).

2.4. Transmission electron microscopy (TEM) to visualize lipid droplets

The quality and morphology of isolated LDs were examined by TEM using positive staining methods. To fix the LDs, the grid was placed onto a drop of 2.5% glutaraldehyde solution (0.1 M PBS, pH 7.2) for 10 min followed by the addition of a drop of 2% osmium tetraoxide solution (0.1 M PBS, pH 7.2) which was incubated for 10 min. Then, the LDs were stained with 0.1% tannic acid for 10 min followed by 2% uranyl acetate for 10 min. The positive stained LDs appeared dark due to the direct contact with osmium tetraoxide. Images and LD diameters were analyzed by Fiji, and the LD diameters were statistically analyzed using Origin Pro 9.0 (OriginLab Corporation).

2.5. In-Solution/Gel Digestion and LC-MS/MS analysis of the lipid droplet proteome

LDs were isolated from two female Macaca mulatta, one female and one male human, and twenty rats. Proteomic analysis of LD proteins from each species was performed one time (due to limited samples) as previously described [15, 25]. Briefly, the LD protein pellet was dissolved in 20 µl of freshly prepared 8M urea or was dissolved in 2 x sample buffer, separated by SDS-PAGE and visualized by silver staining. The indicated bands were sliced out, reduced with 10 mM DTT at 56°C for 1 h, and treated with 40 mM iodoacetamide in the dark for 45 min to block the sulfhydryl groups. Trypsin was added to a ratio of 1:10 (1:20–40

for silver staining) relative to total protein content, and samples were incubated at 37°C overnight. After 12 h, formic acid was added to terminate the enzymatic reaction. The samples were then dried using a speed vacuum. Additional formic acid was added to dissolve the samples. The peptide mixtures were analyzed using an HPLC system coupled to a linear ion trap mass spectrometer LTQ ion trap (Thermo Fishier Scientific). MS/MS data were analyzed using SEQUEST algorithm (v2.8) against Ensemble human (GRCh37.74)/rat (Rnor_5.0.74)/macaque (MMUL 1.0) with Proteome Discoverer (1.4.0.288). All of the searches were performed using a precursor mass tolerance of 3 Da calculated using average isotopic masses. The filter settings for peptides were as follows: enzyme, Trypsin; Max. Missed Cleavage Sites, 2; Min. Peptide Length, 6; Fragment Mass Tolerance, 0.6 Da; Use Average Precursor Mass; Dynamic Modification, methionine oxidation + 15.995 Da; Static Modification, cysteine carbamidomethyl modification + 57.021 Da; q value less than 1%.

2.6. Steroid Labeling

Steroidogenesis was detected by adding 1 μCi [7-^{3}H(N)]-Pregnenolone (PerkinElmer) per sample with/without HSD3B2 inhibitor trilostane (Selleck) for 6 hours on ice followed by incubation with the indicated samples (LDs from adrenal or Hela cells with cytosol or Buffer B) at 37°C for 1 hour. Following the reaction, the mixture was transferred into fresh tube.

Steroids were extracted from the mixture by adding 500 µl chloroform and 200 µl methanol. The samples were separated by Thin Layer Chromatography (TLC) (Yantai Jiangyou silicone company, China). Cold pregnenolone and cold progesterone were added and separated with the extracted lipids from the *in vitro* reaction systems to help locate each steroid. The separation was performed using a chloroform:acetone (75:5) solvent system, and the bands were visualized by exposing TLC plates to iodine. Specific steroids were identified according to standards, were scraped into tubes, and were quantified using liquid scintillation counting (PerkinElmer). Steroid conversion was calculated as the ratio of pregnenolone or progesterone to the sum of the pregnenolone and progesterone.

2.7. Plasmid construction and HSD3B2-GFP site-directed mutagenesis

HSD3B2-Myc/His (pcDNA3.1A+), HSD3B2-EGFP (pEGFP-N1) and retroviral HSD3B2-Myc/His (pQCXIP) plasmids were constructed with human cDNA. The GFP fragment from pEGFP-N1 was cloned and ligated into the retroviral plasmid pQCXIP. PLIN2-Flag (pQCXIP), HSD17B11-Flag (pQCXIP) and HSD17B11-EGFP (pEGFP-N1) plasmids were constructed with mouse cDNA. pDsRed2-Mito was from Clontech. KDEL-Red, PLIN2-dSRed and PLIN1-mCherry were gifts from Peng LI (School of Life Science, Tsinghua University, China).

2.8. Cell culture, transfection, retroviral infection, and stable cell line selection

CHO and AML12 cells were maintained in DMEM/F12 (Macgene Biotech), Hela, 3T3-L1, and 293T cells were maintained in DMEM (Macgene Biotech), and Y1 cell (purchased from National Infrastructure of Cell Line Resource) was maintained in RPMI 1640 Medium (Thermo) that had been supplemented with 10% FBS (Hyclone), 40 μg/ml, 100 U/mL penicillin, and 100 mg/mL streptomycin (Macgene Biotech). The cells were incubated at 37°C with a 5% CO_2 atmosphere. For transient transfections, cells were nucleoporated according to the standard Amaxa programs. For retroviral production, 293T cells were transfected with HSD3B2-Myc/His-pQCXIP, PLIN2-Myc/His-pQCXIP or GFP-pQCXIP plasmids along with envelope and gag-pol plasmids using Lipofectamine 2000 (Invitrogen). The transfections were performed when cells had reached 70-80% confluency and the retroviral supernatant was harvested 48 h after transfection. Hela cells were infected with retrovirus with polybrene (Sigma). Stable cell lines were selected with 1 μg/ml puromycin (Invitrogen).

2.9. Confocal microscopy

Cells transfected by nucleoporation were incubated for 8-12 hours and were then further incubated for 12 hours with/without 200 μM sodium oleate (OA) as previously reported [26]. The cells were fixed in 4% PFA, and LDs were stained with LipidTox Deep Red, for an additional 20 min before mounting. Images were acquired using an inverted microscope (Zeiss, LSM 710). The localization profiles were analyzed with Fiji-ImageJ, a line was marked on LD or ER or mitochondria, and use "Plot Profile" to measure the gray value of each fluorescent protein. If two fluorescent protein show similar profile they are colocalized [27].

2.10. Western blotting

Stable cell lines were harvested in 2 x sample buffer (125 mM Tris-HCL, pH 6.8, 20% glycerol, 4% SDS, 4% β-mercaptoethanol, and 0.04% bromophenol blue). After sonication and centrifugation, supernatants were collected. Equal amounts of protein were separated by SDS PAGE. For immunoblotting, we used primary antibodies (1:2,000) and secondary antibodies (ZSGB-BIO) (1:5,000) in 3% nonfat milk. CGI-58 antibody was gift from Dr. Osumi [28], PLIN2 antibody was from Abcam, HSD3B2 antibody was from Thermo and Abclonal, Rab5, Bip, GFP, Myc, Calnexin antibodies were from Cell Signaling Technology,

Tim23 antibody was from BD Biosciences, GAPDH was obtained from Calbiochem, and Flag antibody was from Sigma. The bands were detected using the Western Lightning Plus ECL system (PerkinElmer).

2.11. Statistical Analysis

Data from at least three independent biological replicates were subjected to statistical analyses using Prism 6.0 or Origin Pro 9.0. Results were expressed as the mean ± SEM as indicated in the figure legends. Comparisons between groups were performed using one-way ANOVA or Student t-tests as appropriate. Proteomic data were analyzed using "R Language", Venn Diagram was drew by using package (VennDiagram) [29].

3. Results

3.1. Some steroidogenic enzymes are present on adrenal lipid droplets

Adrenal LDs from Macaque monkey (M), human (H), rat (R), and castrated swine (S) were isolated by gradient centrifugation, and the isolated LDs were examined by transmission electron microscopy (TEM) and fluorescence microscopy, and observed that isolated LDs were largely intact and spherical (Figure 1A). The diameters of the LDs were distributed

from 200 nm to 2 μm, and a Gaussian fit demonstrated that most were 500 nm to 1000 nm (Figure 1B). Thin-layer chromatography (TLC) analysis of isolated adrenal LDs from all four organisms showed that adrenal LDs contained mainly cholesterol esters (CEs) with relatively little triacylglycerols (TAGs) (Figure 1C). This distinguished them from brown adipose tissue LDs which contained exclusively TAG (Figure 1C), consistent with previous findings [1, 17, 18].

The cholesterol esters of adrenal LDs are primary precursors for steroid hormone synthesis. To investigate whether steroidogenic enzymes may present on LDs, we analyzed the isolated adrenal LD proteins from a female monkey (*Macaca mulatta)* (M1) (Figure 1D). The total isolated LD proteins were separated by SDS-PAGE, revealing three distinct bands at ~55, ~43, ~34 kDa. The ~43 kDa band was particularly prominent in the Colloidal blue stain compared with total membrane (TM), cytosol (Cyto), and whole cell lysate (WCL) (Figure 1D). To exclude the possibility of contamination from other organelles during LDs isolation, monkey LDs were further analyzed by Western blotting for various protein markers of cellular organelles (Figure 1E). As expected, the LD resident protein PLIN2 [30] was exclusively detected in the LD fraction with a similar distribution of the protein RAB5 (Figure 1E), which was previously identified on LDs [15]. Neither the mitochondrial inner membrane protein TIM23 nor the cytosolic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected in the LD fraction (Figure 1E). The endoplasmic

reticulum (ER) luminal protein Bip/GRP78 was found in both the TM and LD fractions (Figure 1E), suggesting that there might be somewhat ER contamination. This result has been reported previously and may be due to physiological interactions between these organelles [15, 31, 32].

To confirm this LD protein profile, we further isolated additional adrenal LD proteins from four organisms, including another female monkey (M2), a female patient with adrenal cancer (H), female rats (R), and castrated swine (S) (Figure 1F). Consistently across these preparations, silver staining of adrenal LD proteins demonstrated three major protein bands at ~55 kDa, ~43 kDa, and ~30 kDa (Figure 1F). To identify these highly abundant proteins in adrenal glands, the three major protein bands from M1 LD protein silver staining gel were excised and subjected to proteomic analysis. Intriguingly, the primary proteins identified were mainly key enzymes involved in steroid hormone biosynthesis, including 3β-hydroxysteroid dehydrogenase (HSD3B2), steroid 17α-hydroxylase 17, 20 lyase (CYP17A1), estradiol 17β-dehydrogenase 11 (HSD17B11), and steroid 21-hydroxylase (CYP21A2), as well as two members of the perilipin family proteins, PLIN2 and PLIN3 (Table 1 and Figure 1G). The LD localization of many of these proteins was subsequently verified by Western blot analysis of LDs from adrenal glands of different organisms (Supplemental Figure S1). Among the proteins identified on the LD, HSD3B2, which catalyzes pregnenolone to progesterone, had the highest score (Table 1). The *Macaca* HSD3B2 is a 42 kDa protein,

consistent with the most heavily stained band at ~43 kDa (Figure 1D and 1F). Western blot analysis of HSD3B2 demonstrated that it presented in the adrenal LD fraction of female *Macaca mulatt*a (M1) (Figure 1E), as well as that of other three organisms and mouse adrenal cortex cell line Y-1 (Supplemental Figure S1), suggesting that HSD3B2 is a LD associated protein. A comparison of the relative intensities of HSD3B2 and Bip and Calnexin in the LD and TM fractions excludes the possibility of this conclusion being due to ER contamination (Figures 1E and Supplemental Figure S1).

3.2. Proteomes reveal distinct steroidogenic enzymes present on various adrenal lipid droplets.

To fully characterize the proteomes of adrenal LDs, we used shotgun mass spectrometry to analyze adrenal LDs isolated from female and male adrenal cancer patients (H), female macaques (M1) and female rats (R). Consequently, 254 proteins were consistently identified on both female and male human adrenal LDs, and 143 proteins on both human and macaques adrenal LDs (Table 2, Supplemental Table 1, and Supplemental Figure S2). Subsequently, all detected steroid synthesis enzymes were mapped to the "diagram of the pathways of human steroidogenesis" (Figure 1G) [33]. The resulting proteomes confirmed that HSD3B and HSD17B11 were easily detected in adrenal LDs of all four organisms (Table 2 and Figure

1G). CYP17A1 was present in all preparations but the adrenal LDs of female rat (Table 2 and Figure 1G). It was previously found that only CYP21A2 is expressed in human being, whereas CYP21A1 is expressed in mice [34]. Consistent with that finding, we detected CYP21A2 only on LDs from human and macaques, and CYP21A1 only on rat LDs (Table 2 and Figure 1G). Interestingly, CYP11B2 was detected in the proteome of rat, which lacked CYP11B1. Other steroidogenic enzymes as well as proteins involved in the intracellular processing and utilization of cholesterol for biosynthesis of sterol hormones were also detected in the proteome of adrenal LDs (Table 2 and Figure 1G). These results suggested that certain steroidogenic enzymes may actually localize on adrenal lipid droplets, but displaying variation in species.

3.3. HSD3B2 localizes on lipid droplets

The biosynthetic pathway from cholesterol to pregnenolone and subsequent catalysis to progesterone by HSD3B2 in the ER or mitochondria is well established [35]. Previously, we showed that HSD3B2 was highly abundant in adrenal LDs (Figure 1E, and Supplemental Figure S1, Table 1 and 2). To confirm the LD localization of HSD3B2 by an independent method, we expressed C-terminal GFP tagged HSD3B2 in Y-1 cell (Figure 2), differentiated 3T3-L1 adipocyte (Supplemental Figure S3), hepatocyte AML12 cell (Supplemental Figure

S4), Hela cell (Supplemental Figure S5) and CHO cell (Supplemental Figure S6). The HSD3B2-GFP fluorescence illuminated rings around LipidTox stained spheres and colocalized with the LD marker protein Plin2-DsRed or Plin1-mCherry, and they showed similar localization profile (Figure 2 and Supplemental Figure S3-S6). The HSD3B2-GFP was also colocalized with ER marker KDEL-Red when it was not on LDs in all cell lines, and in some cell lines HSD3B2-GFP was also observed to colocalize with mitochondrial markers, and Mito-DsRed (Figure 2 and Supplemental Figure S3-S6), which is consistent with previous findings [35, 36]. There was no obvious LD localization of HSD3B2-GFP in Hela cells under normal growth conditions where only a few small LDs are found. However, OA treatment induced the colocalization of HSD3B2 and PLIN2 accompanied by the accumulation and enlargement of LDs (Supplemental Figure S5). Altogether, these lines of evidence consistently support the conclusion that HSD3B2 is present on LDs, in addition to the ER and mitochondria.

HSD17B11 converts androstane-3α, 17β-diol (3α-diol) to androsterone during steroidogenesis [37]. Similar to HSD3B2, HSD17B11 was also detected on LDs, and colocalized with PLIN2 (Supplemental Figure S1 and S7), consistent with our and other groups' previous reports [15, 38].

3.4. Lipid droplets from HSD3B2 overexpressed cells can convert pregnenolone to progesterone

The localization of steroidogenic enzymes on LDs, especially the highly enriched HSD3B2 protein, raised the question whether LDs *per se* have the ability to convert pregenenolone to progesterone. To answer this question, we established an *in vitro* assay system to measure LD steroid enzymatic activity. We first purified LDs from HeLa cells stably expressing HSD3B2-Myc, GFP, or PLIN2-Myc, and then incubated this preparation with cytosol from a cell line stably expressing soluble GFP (GFP cytosol) and the HSD3B2 substrate (cold and ^{3}H-labeled pregnenolone). After the *in vitro* reaction, we extracted the lipids and proteins, and assessed the HSD3B2-mediated synthesis of radiolabeled progesterone using thin layer chromatography (TLC) and liquid scintillation counting. Indeed, LDs with HSD3B2 (3B2-LDs) could efficiently transform ~85% of the pregnenolone substrate to progesterone in one hour, while LDs from the cytosolic GFP (GFP-LDs) or PLIN2-Myc (PLIN2-LDs) cells showed almost no enzymatic activity (Figure 3A and Supplemental Figure S8A). on the contrary, the HSD3B2 specific inhibitor trilostane (3B2-LDs+Tri) efficiently inhibited the enzymatic reaction (Figure 3A). The HSD3B2 protein was only detected in the LD fractions of the in vitro system (Figure 3B). Furthermore, the LD fraction used in the assay system was highly enriched with HSD3B2 but contained only a slight amount of the ER protein Bip (Figure 3C). To explore if the cytosolic fraction provided obligatory components for the 3B2-

LDs mediated steroid synthesis, we replaced the GFP-cytosol with Buffer B (used in the purification of LDs). No steroid synthesis was detected, demonstrating that some component from the cytosol was necessary for enzymatic activity (Supplemental Figure S8B). These results clearly demonstrate that LDs containing HSD3B2 indeed have the capacity to convert pregnenolone to progesterone.

3.5. Isolated native adrenal lipid droplets can convert pregnenolone to progesterone

Based on the *in vitro* results using LDs from cells overexpressing HSD3B2, we speculated that LDs isolated from the adrenal gland could also convert pregnenolone to progesterone. To test this hypothesis, we purified rat adrenal and liver LDs, and conducted a similar *in vitro* assay, using cytosol from adrenal glands. Adrenal LDs (Ad-LDs) could indeed catalyze the synthesis of progesterone, and minimal synthesis was seen with adrenal cytosol (Ad-Cyto) alone or with liver LDs (Li-LDs) (Figure 4A). Treatment with trilostane efficiently blocked the ability of adrenal LDs to synthesize progesterone (Ad-LDs+Tri) (Figure 4A). These results provide clear evidence that progesterone synthesis from pregnenolone can occur on adrenal LDs.

It should be noted that there was a low level of enzymatic activity detected in the liver LD and adrenal cytosol fractions (Figure 4A). This basal enzymatic activity in the adrenal cytosol

fraction might be due to slight contamination by ER or/and mitochondria membranes as indicated by detection of the ER marker Bip and mitochondrial marker Tim23. The LD marker PLIN2 was not detected in the cytosol (Figures 4B and 4C). Although HSD3B2 was also present at a low level in the liver LD (Figure 4B and 4C), the enzymatic activity of Li-LDs was no different from Ad-Cyto (Figure 4A), suggested that the HSD3B2 detected in liver LDs may possess no or little enzymatic activity.

4. Discussion

Adrenal cortex lipid droplets are rich in esterified cholesterols, which are the main precursors for biosynthesis of steroid hormones. Lipid droplets have been found to be physically associated with mitochondria [24, 39], and ER [22, 40, 41]. Although both the ER marker Bip or Calnexin and the mitochondrial marker Tim23 were detected on adrenal LDs isolated from all four organisms and Y-1 cell line by immunoblots (Supplemental Figure S1); immunoblots of the LD marker PLIN2 (Figure 1E), the ratio of peptide number of PLIN family proteins to that of ER marker protein Bip or mitochondrial maker protein VDAC1 in LD proteomic data from all four organisms (Table 2), all indicating that the isolated organelles were enriched adrenal LDs. Furthermore, the colocalization of HSD3B2 and

PLIN1/PLIN2 on LDs also indicate that the enzymatic activity detected was localized to LDs and was probably not due to contamination by ER or mitochondrial material.

The mitochondria of steroidogenic cells have long been thought of as the site for steroidogenesis because of the presence of steroidogenic enzymes including CYP11A1, CYP11B1, and CYP11B2 [1, 4]. Interestingly, our analysis of adrenal lipid droplets revealed that both CYP11A1 and CYP11B1 are also detected on lipid droplets from human, *Macaca*, and rat (Table 2). CYP11B2 was only expressed on rat LDs (Table 2). A recent proteomic study of primary rat granulosa cells also found sterodiogenic enzymes on LDs, including CYP11A1, HSD3B1, HSD3B2 [18]. CYP11A1 synthesizes pregnenolone from cholesterol and is dependent on the mitochondrial environment since mutants lacking the mitochondrial leader or those targeted to the endoplasmic reticulum are inactive [8]. This is because CYP11A1 activity requires electrons donated from NADPH via the electron transfer chain of ferredoxin reductase and ferredoxin, which are both located in the mitochondria [42]. CYP11A1 was reported to couple with an alternative electron donor, microsomal P450 oxidoreductase [3]. We found that ferredoxin reductase (FDXR) and P450 oxidoreductase (POR) were also present in the adrenal lipid droplet proteome (Table 2) and thus CYP11A1 activity may be supported by ferredoxin reductase or P450 oxidoreductase in adrenal LDs, and idea which requires further exploration. However, there was report that the close apposition of mitochondria and lipid droplets would occur upon ACTH induction, which may

occur upon stress when sacrifice the animals [43]. More sophisticated experiments are needed to prove the localization of steroid enzymes that were previously reported on mitochondria.

It seems likely that LIPA or HSL on LDs may catalyze cholesterol esters to release cholesterol, which may be catalyzed to pregnenolone by LD localized CYP11A1 (P450scc) (Figure 5). Conversion of pregnenolone to progesterone by HSD3B then proceeds in the mitochondria. HSD3B is located in the mitochondria, endoplasmic reticulum (ER), and cytoplasm of bovine adrenal zona glomerulosa cells [44]. HSD3B family members lacking mitochondrial leader peptides and are generally found in the cytosol. However, our results demonstrated that HSD3B2 was highly enriched on isolated LDs and TM (ER or mitochondria) in the adrenals, and only on LDs in the liver (Figure 1E, 2, and 4C). Our *in vitro* experiments using LDs purified from HeLa cells stably expressing HSD3B2 or those isolated from adrenal glands revealed that LDs with HSD3B2 could convert pregnenolone to progesterone (Figure 4A). Inhibition of HSD3B2 with trilostane dramatically decreased this conversion (Figure 3A and 4A). Therefore, we speculate that HSD3B2 (3βHSD) may also catalyze pregnenolone to progesterone directly on LDs (Figure 5). The conversion of pregnenolone to progesterone by native adrenal lipid droplets and not by liver lipid droplets, even though they apparently contained small amounts of 3βHSD is very intriguing. By searching human liver LD proteome [21], we found one member of 3βHSD, HSD3B7, that showed 34% sequence identity with HSD3B2, and when transfected into cultured cells, the

enzyme was active against four 7-alpha-hydroxylated sterols, but did not metabolize several different C19/21 steroids (including pregnenolone) as substrates [45]. The amino acids 126 and 372 of Human HSD3B2 was used to as immunogen to produce the polyclone antibody, which may also recognize HSD3B7.

The confocal imaging of HSD3B2 in different cell lines treated with/without OA confirmed the LD localization of HSD3B2 and HSD17B11. One speculation arising from these observations is that close interactions between LDs and ER or mitochondria may facilitate lipid transfer for the biosynthesis of steroid hormones (Figure 5). The small GTPase RAB18 has been implicated in mediating LD-ER association [31, 41], and is present on LDs from all four organisms (Table 2). Consistent with this hypothesis, previous work showed that redistribution of HSD17B11 from ER to LD might function in the synthesis of steroid hormones [22].

Although the ER and mitochondria have been considered as the major sites of steroidogenesis, this study shows that several steroidogenic enzymes abundantly present on adrenal cortex lipid droplets, which may also serve as sites for steroid hormone metabolism as well (Figure 5).

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Conflict of interests

The authors declare that they have no conflict of interests.

Contributions

Conceived and designed the experiments: B.L, PS L, and JH Y; performed the experiments: JH Y, XT Z; conducted imaging and part MS data analysis: YH L, SM X, HZ W; conducted part radioactive experiment and plasmid construction, LQ Z, XM Z conducted EM. Analyzed

the data: B.L, PS L, JH Y, SM X, and XT Z. Contributed reagents/materials/analysis tools: B.L, PS L, JH Y, HC Z. Wrote the paper: B.L, PS L, JH Y.

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Table 1. LC-MS of three major protein bands of *Macaca mulatta* adrenal LDs

55 kDa-Band Proteins	**MW/kDa**	**Peptide Number**	**Score**	**Coverage**	**PSMs**
Steroid 17-alpha-hydroxylase/17,20 lyase CYP17A1)*	57.6	21	36.49	38.58	27
ATP synthase subunit alpha (ATP5A1)	59.7	11	12.10	28.76	12
PLIN2 (Adipophilin /ADRP)#	48.0	9	24.06	30.34	10
steroid 21-hydroxylase (CYP21A2)*	56.1	7	8.95	21.50	7
43 kDa-Band Proteins					
3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase type 2 (HSD3B2) *	42.0	20	83.97	47.45	53
apoptosis-inducing factor 2 (AIFM2)	40.5	6	11.54	31.37	7
GTPase IMAP family member 4 (GIMAP4)	37.4	4	6.43	20.97	4
PLIN3 (Tip47)#	43.9	3	3.61	18.61	
34 kDa-Band Proteins					
3 beta-hydroxysteroid dehydrogenase/Delta 5-->4-isomerase type 2 (HSD3B2)*	42.0	11	21.44	39.14	13

Estradiol 17-beta-dehydrogenase 11 (HSD17B11) *	28.1	5	10.63	31.64	5
Peroxiredoxin-6 (PRDX6)	25.0	2	6.69	15.18	3

Three protein major bands excised from Figure 1C were analyzed by LC-MS/MS, and detected peptides were blasted against the *Macaca mulatta* protein database to identify major LD proteins, and only proteins with coverage ≥ 15% were shown. Proteins marked with * are involved in steroid biosynthesis, and proteins marked with # are well studied PLIN family proteins. Protein score (Score) is the sum of the highest ions score for each distinct sequence. Peptide-spectrum match score, PSMs.

Table 2. Proteins or enzymes involved in steroidogenesis were dectected in adrenal LD fraction

Protein/enzyme	Description	Female Human	Male Human	Female Monkey	Rat
CYP11A1 (P450scc)	cytochrome P450, family 11, subfamily A, polypeptide 1	19	18	13	18
CYP11B1 (P450c11β)	cytochrome P450, family 11, subfamily B, polypeptide 1	22	22	13	#
CYP11B2 (P450c11AS)	cytochrome P450, subfamily 11B, polypeptide 2	#	#	#	5
CYP17A1 (P450c17)	cytochrome P450, family 17, subfamily A, polypeptide 1	32	26	26	#

CYP21A1 (P450c21)	cytochrome P450, subfamily 21A, polypeptide 1	#	#	#	17
CYP21A2 (P450c21)	cytochrome P450, family 21, subfamily A, polypeptide 2	11	12	9	#
HSD3B1(3βHSD1)	3 beta-hydroxysteroid dehydrogenase 1	#	#	#	15
HSD3B2(3βHSD2)	3 beta-hydroxysteroid dehydrogenase 2	16	12	15	#
HSD11B2(11βHSD2)	11-beta hydroxysteroid dehydrogenase 2	#	#	#	6
HSD17B7(17βHSD7)	17-beta hydroxysteroid dehydrogenase 7	8	8	11	5
HSD17B11(17βHSD11)	(17-beta) hydroxysteroid dehydrogenase 11	20	13	14	8
PLIN1	Perilipin 1	7	2		
PLIN2	Perilipin 2	20	13	8	22
PLIN3	Perilipin 3	14	13	13	2
Bip/GRP78	Heat Shock Protein Family A (Hsp70) Member 5	4	7	2	4
HSL	hormone sensitive lipase				8
LIPA	lipase A, lysosomal acid, cholesterol esterase	4	#	#	2
ACAT1	acetyl-Coenzyme A acetyltransferase 1	4	9	#	2
FDXR	ferredoxin reductase	14	14	2	13
POR	P450 (cytochrome) oxidoreductase	10	9	2	11
NPC1	Niemann-Pick disease, type C1	9	#	#	#

NPC2	Niemann-Pick disease, type C2	2	#	#	#
STAR	steroidogenic acute regulatory protein	2	#	#	3
VDAC1	voltage-dependent anion channel 1	6	6	4	6
VDAC2	voltage-dependent anion channel 2	6	5	#	#
VDAC3	voltage-dependent anion channel 3	4	4	#	4
RAB18	RAB18, member RAS oncogene family	5	5	6	3

Adrenal LDs from four organisms were isolated. LD proteins from humans (one female patient and one male patient), female *Macaca mulatta*, and female rat adrenals were analyzed by shotgun LC-MS/MS. Proteins involved in steroid hormone synthesis are shown. The number indicated unique peptide hit numbers, # indicated non-detected.

Figure Legends

Figure 1. Isolation of adrenal lipid droplets from four organisms

(A) Purity of adrenal LDs. Adrenal LDs from female humans were subjected to microscopic analysis, including Transmission Electron Microscopy (TEM) positive staining (left),

LipidTox Red staining (middle), and Differential Interference Contrast (DIC) (right). The scale bar represents 10 μm.

(B) The distribution of lipid droplet sizes (diameter).

(C) Adrenal LDs mainly contain cholesterol esters (CE). LD lipids of male mouse brown adipose tissue (BAT) and adrenals from female *Macaca mulatt*a (M), female human (H), female rats (R) and castrated swine (S) were analyzed by thin-layer chromatography (TLC) with the following lipid markers: cholesterol esters (CE), triacylglycerol (TAG), two isoforms of diacylglycerols (DAG, DAG1 is 1,3-DAG, DAG2 is 1,2-DAG), and phospholipids (PL, phosphocholine and phosphoethanolamine).

(D) Visualization of female *Macaca mulatt*a (M1) adrenal LD proteins by Colloidal Blue staining of SDS-PAGE gels, with equal protein loaded in each lane. (lipid droplets, LD; total membrane, TM; cytosol, Cyto; whole cell lysate, WCL).

(E) Western blot analysis of female *Macaca mulatt*a (M1) adrenal lipid droplet proteins. Equal amounts of protein extracted from LDs (LD), total membrane (TM), cytosol (Cyto) and whole cell lysate (WCL) were separated by SDS-PAGE, transferred to a membrane and were probed with different cellular organelle markers: LD marker, PLIN2 and RAB5; mitochondrial marker, TIM23; endoplasmic reticulum (ER) marker, Bip/GRP78; cytosol marker, GAPDH.

(F) Silver staining of LD proteins from female *Macaca mulatta* (M1 and M2), female humans (H), female rats (R), and castrated swine (S). Arrowheads indicated the three major protein bands of M1 were sliced and subjected to proteomic analysis.

(G) Detected steroid synthesis enzymes by proteomics analysis of LD proteins from female human, male human, female monkey and female rat adrenal glands. Enzymes with blue colored background indicated detection by both cut gel and short-gun proteomics; enzymes with orange colored background indicated detection only by short-gun proteomics; enzyme with green colored background indicated no detection. Enzymes with * indicated only detected in primate adrenal glands; # indicated only detected in rat adrenal glands.

Figure 1

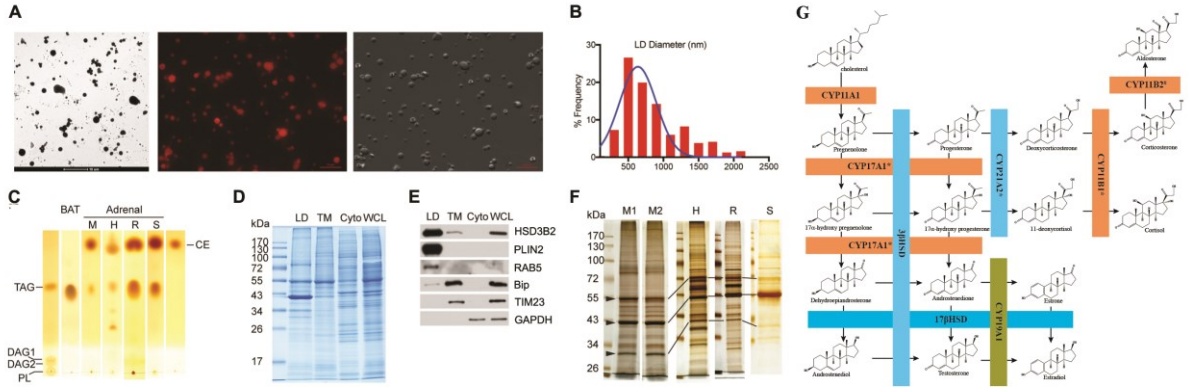

Figure 2. HSD3B2 localized on LDs in Y-1 cells

Cellular localization of HSD3B2. Y-1 cells were transfected with GFP-tagged human HSD3B2 and either the LD marker Plin2-dSRed and Plin1-mCherry, the ER marker KDEL-Red, or the mitochondrial marker Mito-dSRed2. The localization of HSD3B2 was observed in cells. LDs were stained with LipidTox Deep Red (LipidTox-DR). The first column of figures presents the different organelle makers, the second column is HSD3B2-GFP, the third column is LipidTox Deep Red stained LDs, the third column is the merged image, and the last column is the localization profile of each fluorescent protein. Scale bar represents 10 μm.

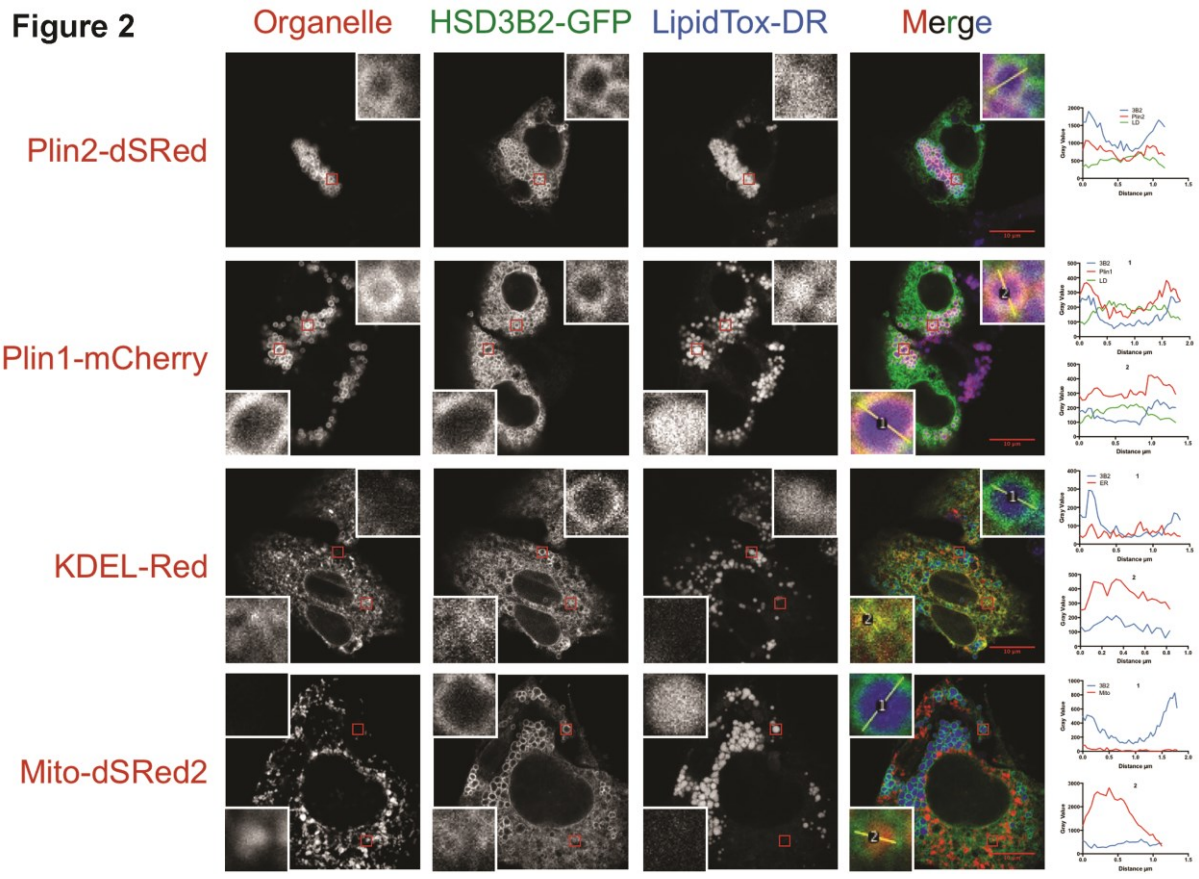

Figure 3. LDs isolated from Hela cells stably expressing HSD3B2-Myc convert pregnenolone to progesterone *in vitro*

(A) Quantitation of the ratio of radioactive pregnenolone and progesterone to the sum of these two steroids (Pre or Pro ratio) after *in vitro* reactions. Cytosol from HeLa cells stably expressing GFP (GFP cytosol) was mixed with LDs isolated from HeLa stably expressing HSD3B2 (3B2-LDs) or GFP (GFP-LDs). The mixtures were incubated with cold and radioactive pregnenolone for *in vitro* progenterone production. The quantity of progesterone produced by 3B2-LDs was significantly greater than that of 3B2-LDs treated with the HSD3B2 inhibitor trilostane (3B2-LDs + Tri), GFP-LDs, and GFP cytosol. The results represent the mean ± SEM from triplicate samples. The * symbol indicates the statistical significance of progesterone between each group, ** $p < 0.01$; The $^{\$}$ symbol indicates the statistical significance of pregnenolone between each group, $^{\$\$}$ $p < 0.01$.

(B) The components of the *in vitro* steroid synthesis system were detected with specific protein markers: HSD3B2 protein, PLIN2 (LD marker), GAPDH (cytosolic marker), Bip (ER marker). All lanes were loaded with equal protein.

(C) HSD3B2 was enriched in LDs fraction isolated from HeLa cells stably expressing HSD3B2-Myc but not in those from cells stably expressing GFP. The lanes were loaded with equal protein from each fraction and probed with antibodies as follow: PLIN2 (LD marker),

Bip (ER marker), TIM23 (mitochondrial marker), and GAPDH (cytosolic marker).

Abbreviations: total membrane, TM; cytosol, Cyto; whole cell lysate, WCL.

Figure 3

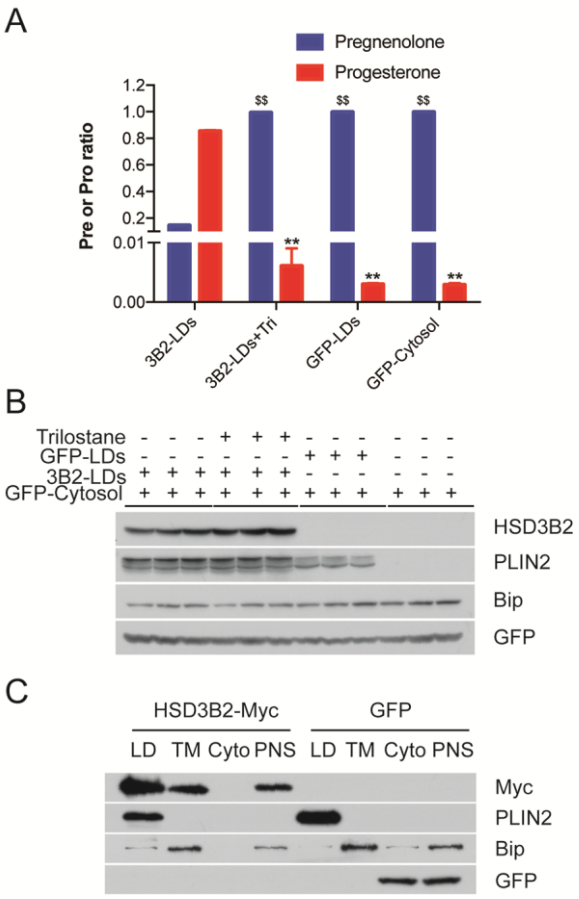

Figure 4. Adrenal LDs convert pregnenolone to progesterone *in vitro*

(A) Quantitation of the ratio of radioactive pregnenolone and progesterone to the sum of these two steroids (Pre or Pro ratio) after *in vitro* reactions. Rat adrenal cytosol (Ad-Cyto) was mixed with LDs isolated from adrenal gland (Ad-LDs) or liver (Li-LDs) (experimental group) or with no LDs (negative control). The mixture was incubated with cold and radioactive pregnenolone for *in vitro* progesterone production. The quantity of progesterone produced by adrenal LDs was significantly greater than that of adrenal LDs treated with the HSD3B2 inhibitor trilostane (Ad-LDs + Tri), Li-LDs, and, Ad-Cyto. The results represent the mean ± SEM from triplicate samples. The symbol * indicates the statistical significance of progesterone between each group, ** $p < 0.01$; The symbol $^{\$}$ indicates the statistical significance of pregnenolone between each group, $^{\$\$}$ $p < 0.01$.

(B) The components of *in vitro* steroid synthesis system were detected with specific protein markers: PLIN2 (LD marker), GAPDH (cytosol marker), Bip (ER marker) and Tim23 (mitochondrial marker). All lanes were loaded with equal protein.

(C) HSD3B2 was detected both in isolated adrenal and liver LD fractions. The lanes were loaded with equal protein from each fraction and probed with antibodies as follow: PLIN2 (LD marker), Bip (ER marker), Tim23 (mitochondrial marker), and GAPDH (cytosolic marker). Abbreviations: Total membrane, TM; cytosol, Cyto; whole cell lysate, WCL.

Figure 4

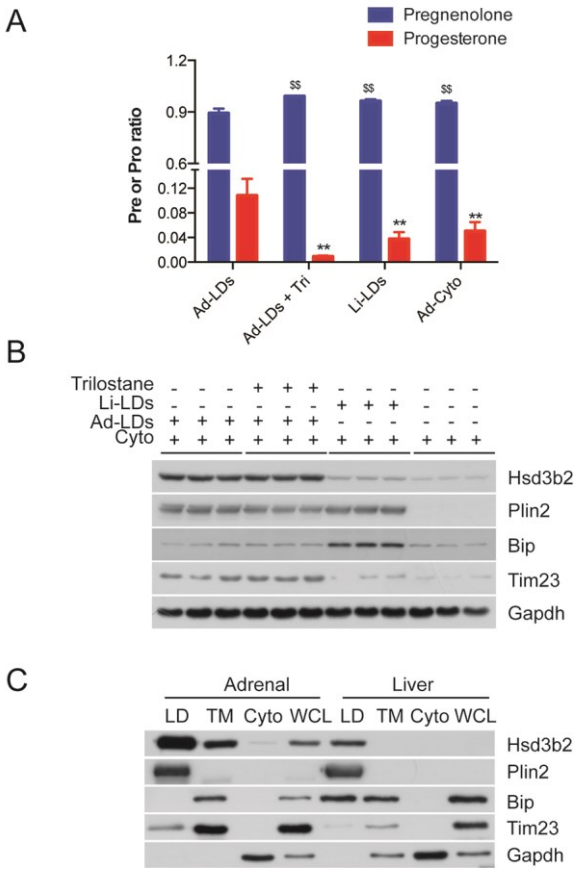

Figure 5. A proposed model of steroid hormones metabolism

Cholesterol originating from LDs, *de novo* synthesis, or extracellular lipoproteins, is transported into mitochondria via steroidogenic acute regulatory protein (StAR), and is then catalyzed to steroid hormones by different steroidogenic enzymes residing in mitochondria (The classical pathways indicated by blue arrows, reviewed by Walter L. Miller [3]). Alternatively, steroid hormones metabolism may also occur on LDs directly (indicated by pink arrows). Cholesterol esters in LDs are hydrolyzed to cholesterol by lysosomal acid lipase A (LIPA) or hormone-sensitive neutral lipase (HSL). Then, cholesterol is probably directly catalyzed to steroid hormones by various steroidogenic enzymes present in abundance on LDs.