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Adrenal 20α-Hydroxysteroid Dehydrogenase in the Mouse Catabolizes Progesterone and 11-Deoxycorticosterone and Is Restricted to the X-Zone

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The enzyme 20α-hydroxysteroid dehydrogenase (20α-HSD) is a progesterone-catabolizing enzyme that is highly expressed in mouse ovaries and adrenals. Although the functional significance of ovarian 20α-HSD for the induction of parturition has been defined, regulation and distribution of 20α-HSD in the adrenal gland has not been determined. We demonstrate that the expression of adrenal 20α-HSD is restricted to the X-zone, a transient zone between the adrenal cortex and the medulla of yet unknown function. Adrenal 20α-HSD activity in male mice peaks at 3 wk of age and disappears thereafter, whereas 20α-HSD enzyme activity is maintained in adrenals from nulliparous female animals. Testosterone treatment of female mice induces rapid involution of the X-zone that is associated with the disappearance of the 20α-HSD-positive cells. Conversely, reappearance of 20α-HSD expression and activity in male animals is evident after gonadectomy. Moreover, pregnancy, but not pseudopregnancy, is accompanied by X-zone regression and loss of 20α-HSD activity. Pregnancy-induced X-zone regression and -abolished 20α-HSD expression is partially restored in animals that were kept from nursing their pups. We found that in addition to its progesterone-reducing activity, 20α-HSD also functions as an 11-deoxycorticosterone-catabolizing enzyme. The unaltered growth kinetics of the X-zone in 20α-HSD knockout animals suggests that 20α-HSD is not required for the regulation of X-zone growth. However, 20α-HSD expression and enzymatic activity in all experimental paradigms is closely correlated with the presence of the X-zone. These findings provide the basis for 20α-HSD as a reliable marker of the murine X-zone. (*Endocrinology* 148: 976–988, 2007)

THE STEROIDOGENIC ENZYME 20α-hydroxysteroid dehydrogenase (20α-HSD) has been initially described as a progesterone-metabolizing enzyme of the ovary (1). On a functional level, ovarian 20α-HSD is actively involved in the control of progesterone homeostasis in pregnancy of rats and mice (2, 3). Although 20α-HSD expression and activity is down-regulated in the corpus luteum of pregnancy, 24 h before parturition, ovarian 20α-HSD activity is acutely stimulated (4). Accordingly, in mice with targeted deletion of the 20α-HSD gene, progesterone blood concentration remains high throughout pregnancy, which results in a delay of 2–4 d in parturition (3). These findings indicate that expression of 20α-HSD activity is mandatory for the induction of parturition through reduction of progesterone blood concentration.

In mice, extra-ovarian presence of 20α-HSD has been identified in immune cells and the kidney (5, 6), and high 20α-HSD activity has been located to the adrenal gland (7, 8). However, the regulation of enzymatic activity and functional significance of this tissue-restricted expression pattern remains elusive.

The primate adrenal cortex is characterized by three morphologically distinct concentric zones, the outer zona glomerulosa, the intermediate zona fasciculata, and the inner zona reticularis. These zones have also defined functional properties, with mineralocorticoids being synthesized in the zona glomerulosa, glucocorticoids being produced in the zona fasciculata, and adrenal androgens being secreted from the zona reticularis (reviewed in Ref. 9). The adrenal glands of mice and rats lack a functional distinct zona reticularis and do not synthesize androgens because of the absence of 17α-hydroxylase (CYP17) expression (9). However, adrenals of both rodents and primates posses a transient zone between the adrenal cortex and the adrenal medulla: the murine X-zone and the human fetal zone. Under normal circumstances, the human fetal zone, which is required to establish the intrauterine estrogenic milieu of pregnancy, undergoes apoptosis soon after birth (10). The murine X-zone develops after birth and regresses at puberty in the male (11) and during the first pregnancy in the female animal (12). Although the timing of development of the fetal zone in humans and the X-zone in mice differs, several lines of evidence suggest that these two zones are analogous structures. They are located in the same position adjacent to the adrenal medulla, and they both have ultrastructural features of steroid-producing cells (13). Moreover, transgenic expression of lacZ driven by a specific SF-1 promoter fragment, demonstrates

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Abbreviations: DHT, Dihydrotestosterone; DOC, 11-deoxycorticosterone; dpc, day post coitum; HPF, high-power field; HSD, hydroxysteroid dehydrogenase; 20α-OHDOC, 4-pregnen-20α,21-diol-3one; OHP, hydroxyprogesterone; PGF2α, prostaglandin F2α; StAR, steroid acute regulatory protein.

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maintenance of lacZ staining from the mouse fetal adrenal gland only in the X-zone (14), indicating that the X-zone indeed represents remnants of the adrenal primordia that forms before the formation of the definite zone. Furthermore, loss of function of the Dax-1 gene (dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on the X chromosome) in humans (15) and mice (16) results in lack of regression of the fetal and the X-zone, respectively.

The adrenal cortex is a dynamic organ in which constant replacement with newly differentiated daughter cells allows for the plasticity to respond to the needs of the organism. Each zone of the adrenal cortex is derived from a common pool of progenitor cells located in the periphery of the cortex, which migrate centripetally and populate the inner cortical zones upon differentiation (17, 18), although the possibility exist that X-zone cells may originate from the mouse adrenal fetal zone (14). Thus, in addition to transcriptional regulation of steroidogenic enzymes as evident with ACTH-induced up-regulation of steroid acute regulatory protein (StAR) and side-chain cleavage enzyme (19), adrenal steroid output can be further regulated by changes in the zonal composition through growth or regression of adrenocortical zones that harbor specific steroidogenic properties. However, what factors regulate zone-specific growth dynamics is largely unknown. Moreover, the overall function of the X-zone remains unclear. In the mouse adrenal, the presence of 20α-HSD RNA transcripts (8) and enzymatic activity (20) have been reported, and it has been suggested that the pattern of adrenal 20α-HSD expression could be dependent on the presence of the X-zone (21). Based on the parallel loss of 20α-HSD enzymatic activity and regression of the adrenal X-zone, we hypothesized an X-zone-restricted adrenal expression of 20α-HSD. Furthermore, we assessed the possibility that in addition to progesterone, other adrenal steroids could serve as a substrate of 20α-HSD. Thus, we used an integrated approach that combined histology, immunohistochemistry staining, enzymology, and molecular biology to study the dynamics of 20α-HSD-expressing cells within the adrenal in response to hormonal stimulations *in vivo* and *in vitro*.

Materials and Methods

Measurement of 20α-HSD enzymatic activity

Reduction of progesterone to 20α-hydroxyprogesterone (20α-OHP). The progesterone-catabolizing enzyme activity of 20α-HSD was determined essentially as described earlier (5, 6). Tissues were disrupted in glass Teflon homogenizers in PBS, the homogenate was centrifuged at 13,000 rpm for 10 min, and the supernatants were analyzed for 20α-HSD activity (conversion of the substrate progesterone to 20α-OHP). The measurement of enzymatic activity was performed in glass tubes, containing 3–10 μg protein in 0.25 ml reaction mixture consisting of 0.01 M sodium phosphate (pH 7.4), 1.0 mM $MgCl_2$, 10^{-5} M progesterone, 0.2 μCi [^{3}H]progesterone (NEN Life Science Products, Boston, MA; 102 Ci/mmol), and a NADPH regenerating system consisting of 1.0 mM NADPH, 10 mM glucose-6-phosphate, and 2.0 U/ml glucose-6-phosphate dehydrogenase (reagents from Sigma Chemical Co., St. Louis, MO). The incubation period was 30–40 min at 37 C, and reactions were stopped by addition of 2 ml diethyl ether containing 1 μg progesterone and 1 μg 20α-OHP as carriers. The tubes were vortexed to extract the steroids into the organic phase, which was subsequently separated and evaporated overnight in a chemical hood. The extracted steroids were separated on fluorescent silica gel 60 F 254 plates (Merck, Darmstadt, Germany) in a diethyl ether/chloroform 3:10 (vol/vol) system. Radioactive progesterone and 20α-OHP were visualized and quantified by phosphoimaging on a Personal Molecular Imager FX (Bio-Rad, Hercules, CA). Results were calculated as the amount of picomoles 20α-OHP generated per hour per microgram protein.

Reduction of 11-deoxycorticosterone (DOC) to 4-pregnen-20α,21-diol-3one (20α-OHDOC). The reaction mixture was used as described above, whereas progesterone and radioactive progesterone were replaced by 10^{-5} M DOC (Sigma). Adrenal tissue homogenates were prepared as described, but 60 μg protein was added to the reaction mixture and the progesterone and 20α-OHP carriers were omitted from the diethyl ether that stopped the reaction. The extracted steroids were analyzed and quantified by HPLC (Agilent 1100 series) using the Lichrospher 5U 100A C18 250 × 4.00 (5-μm) column and DAD-240/10 nm, Ref 400/10 detector. The solvent was a gradient of acetonitrile/water from 40–80% acetonitrile. The standards were DOC (Sigma) with a retention time of 8.56 min and 20α-OHDOC and 4-pregnen-20β,21-diol-3one (20β-OHDOC; Steraloids, Newport, RI) with retention times of 7.41 and 8.00 min, respectively. Results were calculated as the amount of picomoles 20α-OHDOC generated per hour per microgram protein. For determination of enzyme kinetics, recombinant 20α-HSD, 0.012 and 0.06 μg recombinant protein, was used for the reduction of progesterone and DOC, respectively.

Mice

BalB/c mice purchased from Harlan (Jerusalem, Israel) were grown at the Ben Gurion University of the Negev animal facility at 22 C in a 12-h light, 12-h dark cycle. The mice had free access to mouse chow and water. The generation of 20α-HSD knockout (20α-HSD −/−) mice has been published previously, and genotyping was determined by PCR as described (3). Unless otherwise specified, BalB/c mice were used. Orchidectomy and sham orchidectomy were performed under anesthesia [ketamine 80 mg/kg body weight plus rompun (Xylazine) 16 mg/kg body weight]. Pseudopregnancy was induced by mating female mice with sterile (vasectomized) males, and pseudopregnancy was confirmed by measurement of progesterone blood concentration (above 25 ng/ml). The day in which the vaginal plug was observed was considered the first day post coitum (dpc). Stat5ab knockout mice (22) were a generous gift from Dr. J. N. Ihle (St. Jude Children Research Hospital Memphis TN). The experimental protocol was approved by the Committee for the Ethical Care and Use of Animals in Experiments of Ben Gurion University of the Negev.

Hormonal treatment

Steroid treatment. Hormone implants were made from SILASTIC brand tubes (inner diameter, 1.98 mm; outer diameter, 3.18 mm; length, 2 cm; Dow Corning, Midland, MI) containing 40 mg steroid (without vehicle) and sealed by SILASTIC brand adhesive (Dow Corning). The implant was inserted under the skin of the back as described before (23), with empty sealed implants serving as controls. For short-time androgen treatment, testosterone propionate (1 mg in 100 μl soybean oil) and 100 μl soybean oil as vehicle was applied sc in the back. Dexamethasone (Merck) in 200 μl PBS and 200 μl PBS as vehicle were applied for 10 d with ip injections of 128-μg daily doses. Sex steroids were purchased from Sigma.

Treatment with Estrumate (cloprostenol), a prostaglandin F2α (PGF2α) agonist. Pregnant mice (15–17 dpc), with the day of the vaginal plug considered as 1 dpc) were injected sc in the upper back with 15 μg Estrumate (Essex Animal Health, Burgwedel, Germany) freshly dissolved in 250 μl PBS with two doses of 250 μl, one at 1000 h and the second dose at 1400 h, whereas control mice received PBS alone (3).

Hormone measurement

Serum progesterone levels were measured using a solid-phase ^{125}I RIA kit (Diagnostic Products Corp., Los Angeles, CA). Female mice were anesthetized with isoflurane (Minrad Inc., Buffalo, NY) and bled through the orbital sinus using micro hematocrit tubes (Brand GmbH, Wertheim, Germany). Sera were recovered after centrifugation at 13,000 rpm for 10 min and frozen at −80 C, and RIA was performed according to the manufacturer's protocol. Serum testosterone levels were mea-

sured by the Laboratory of Endocrinology at the Soroka University Hospital, Beer Sheva, Israel.

Antibodies, SDS-PAGE, and Western blotting

Antibodies against 20α-HSD were raised in rabbits using a peptide from murine 20α-HSD (amino acids 294–313, KILDGLDRNLRYFPADMFKA) coupled to keyhole limpet hemocyanin. Tissues were disrupted and homogenized in PBS and centrifuged as above, supernatants mixed with lysis buffer and separated by SDS-10% PAGE and transferred to nitrocellulose. Membranes were probed by the 20α-HSD antiserum (1:3000), anti-StAR antiserum (1:1000; D.M. Stocco, Lubbock, TX) or anti-β-actin antibodies (1:20,000; ICN Biomedicals Inc., Aurora, OH) and visualized with the ECL detection system (Amersham, Piscataway, NJ) as described previously (3).

Histology and immunohistochemistry

One adrenal per animal was rapidly dissected and placed in 4% paraformaldehyde overnight at 4 C, whereas the contralateral adrenal was frozen (−80 C) and kept for enzymatic assay of 20α-HSD. Tissues were dehydrated and embedded in paraffin, and 7-μm sections were cut and stained with hematoxylin and eosin using standard protocols. For immunohistochemistry, paraffin-embedded sections were rehydrated, blocked with 0.3% H_2O_2 in methanol for 10 min, and incubated overnight with a rabbit polyclonal antibody (20α-HSD, 1:2500; 3β-HSD, 1:2500; A. Payne, Stanford, CA) in blocking buffer containing 3% BSA (Roche, Indianapolis, IN), 5% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA), and 0.5% Tween 20. Bound antibody was detected using the Vectastain ABC Kit Standard (Vector Laboratories Inc., Burlingame, CA) according to the manufacturer's protocol.

Synthesis of recombinant 20α-HSD protein

The full-length 20α-HSD cDNA was obtained from EST clone AA105098 (IMAGE 533514 Genome System Inc., St. Louis, MO) and inserted into the pGEX2T expression vector (Amersham Biosciences, Arlington Heights, IL). After transformation in *Escherichia coli* DH5α, expression of the recombinant GST-20α-HSD fusion protein was induced by isopropyl-β-D-thiogalactopyranoside (0.1 mM) for 4 h. Subsequently, cells were pelleted, resuspended in PBS (pH 7.3) with 2% sarcosyl (Sigma), and disrupted by mild sonication after which Triton X-100 was added to a final concentration of 2%. Cell debris was removed by centrifugation at 2500 × *g* for 15min at 4 C. The fusion protein from the supernatant was adsorbed to glutathione agarose (Sigma) overnight and treated with thrombin using an on-column cleavage and purification procedure as recommended by the manufacturer. The Michaelis-Menten constant (K_m) and the maximum velocity of the reaction (V_{max}) for recombinant 20α-HSD enzyme were calculated from turnover of progesterone (1–80 μM) and DOC (2–40 μM) as substrates, respectively, with Hanes Woolf plots using an online web application (http://bioweb.pasteur.fr/seqanal/interfaces/findkm.html).

Statistical analysis

All results are expressed as mean ± SEM. The number of independent experiments is given in the figure legends. Statistical comparisons were analyzed by single-factor ANOVA. Statistical significance is defined as $P < 0.05$.

Results

Time course of adrenal 20α-HSD protein expression and enzymatic activity parallels growth and regression of the X-zone

To define the time course of adrenal 20α-HSD expression, adrenals from male and female mice were harvested at various ages and 20α-HSD protein levels were evaluated by Western blotting. Although 20α-HSD expression was readily detectable in female adrenals at all time points (from 10 d until 17 wk), in male adrenals, 20α-HSD protein expression was evident only around the third week of age (Fig. 1A). In parallel with this expression profile, adrenal 20α-HSD enzymatic activity displayed significant gender differences. In female animals, adrenal 20α-HSD activity was detectable in the first week of life (120.0 ± 1.6 pmol 20α-OHP/μg·h) with a peak activity in the second week (382.0 ± 36.1 pmol 20α-OHP/μg·h; Fig. 1B). At later time points, activity remained high for at least 8 wk and started to decline around the age of 20 wk (169.0 ± 40.5 pmol 20α-OHP/μg·h) and further decreased thereafter with a remaining 25% activity at 1 yr of age (99.9 ± 21.3 pmol 20α-OHP/μg·h; Fig. 1B). In contrast, adrenals from male animals displayed an overall lower enzymatic activity with a peak within the third week of age (224.8 ± 21.9 pmol 20α-OHP/μg·h) and a rapid decrease thereafter with 30% activity remaining at the age of 4 wk (91.6 ± 47.6 pmol 20α-OHP/μg·h) and complete loss thereafter. Interestingly, loss of 20α-HSD activity preceded the rise in serum testosterone levels, indicating the onset of puberty (Fig. 1B).

These results were closely correlated with immunohistochemical staining, indicating X-zone-restricted 20α-HSD expression (Fig. 1C). Accordingly, X-zone regression in the male animal (older than 3 wk) was accompanied by a complete loss of 20α-HSD immunoreactivity, whereas in females, the X-zone and the 20α-HSD-expressing cells within are detected in adult mice (2.5 months old), and a small number of 20α-HSD-positive cells remained in the adrenals from 5-month-old females (Fig. 1C). Taken together, the time course of adrenal 20α-HSD protein expression and enzymatic activity together with zonal distribution of 20α-HSD expression provide additional evidence for an X-zone restriction of adrenal 20α-HSD expression.

Androgens induce X-zone regression and loss of adrenal 20α-HSD activity, which is partially reversed by gonadectomy

Because androgens have been implicated in X-zone regression, we studied effects of testosterone treatment and gonadectomy on 20α-HSD enzymatic activity and expression. When injected in 2-month-old female mice, testosterone propionate led to a rapid drop in both 20α-HSD activity and protein expression after 1 d (inhibition of enzyme activity, 42.2 ± 7.9%; inhibition of protein expression, 65.4 ± 6.2%) and further reduction after 3 d (86.6 ± 6.2%; 97.1 ± 2.7%) and 7 ds (92.6 ± 4.3%; 95.4 ± 4.6%), respectively (Fig. 2A, the reduction in protein expression is not shown in the figure). These changes were paralleled by a loss of 20α-HSD-positive cells as detected by immunohistochemistry (Fig. 2B). In addition to testosterone, we studied the effect of dihydrotestosterone (DHT), which cannot be aromatized to estrogen. Seven days of treatment of female animals with DHT-containing SILASTIC brand capsules resulted in complete regression of the X-zone and elimination of 20α-HSD activity (Fig. 2C, time-point 0). Conversely, removal of DHT implants after 7 d of treatment with DHT-containing Silastic capsules was followed by partial recovery of adrenal 20α-HSD activity (11.2 ± 2.1 pmol 20α-OHP/μg·h at d 0 and 80.8 ± 16.3 pmol 20α-OHP/μg·hat d 28, $P < 0.004$; Fig. 2C), whereas implantation and removal of empty tubes did not

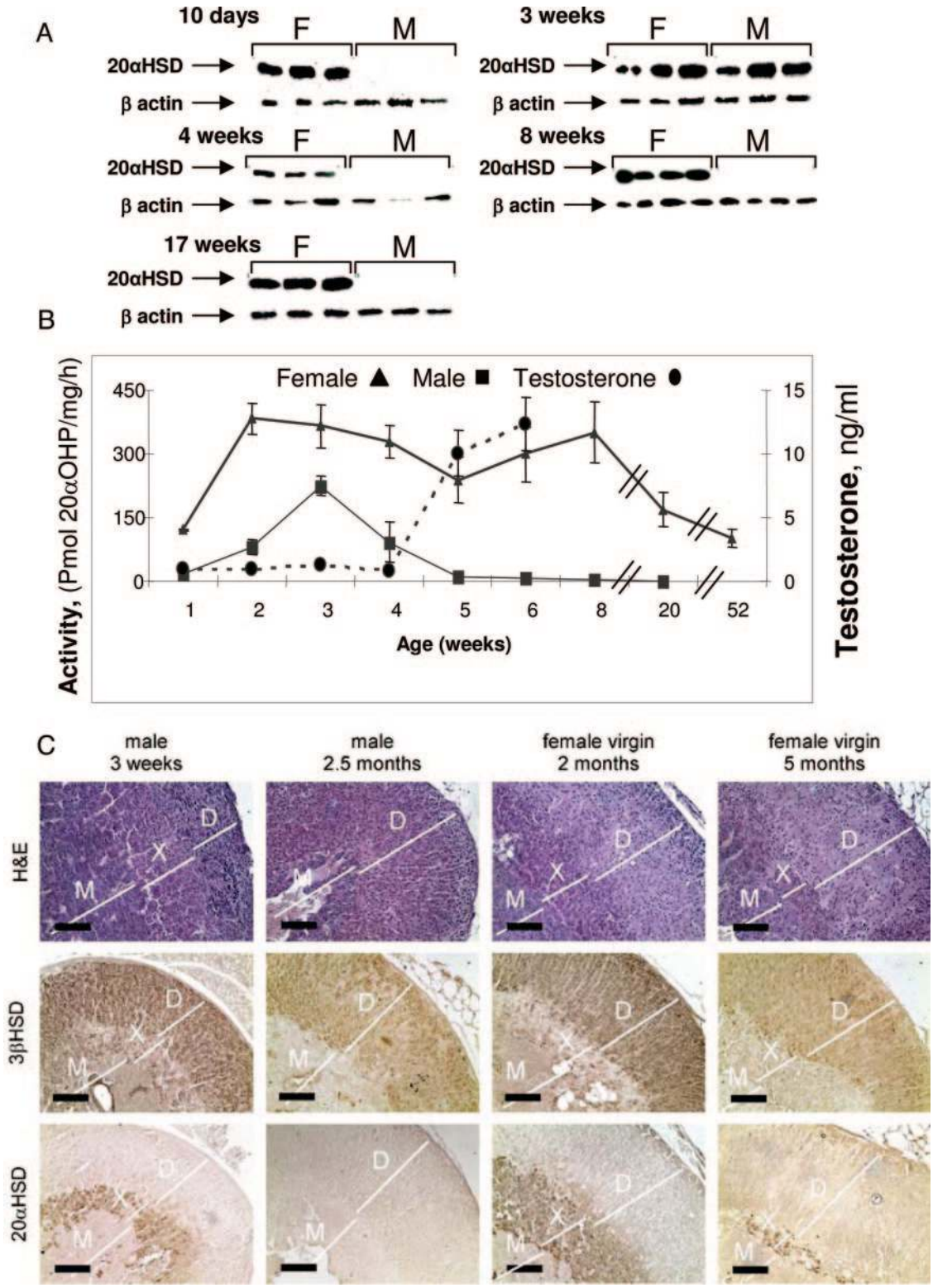

FIG. 1. Time course of adrenal 20α-HSD protein expression and enzymatic activity. Western blot experiments in female and male mice at various ages (n = 3 each) reveal adrenal 20α-HSD expression in all nulliparous female mice up to 17 wk of age, whereas in male animals, 20α-HSD expression is restricted to a time frame before the onset of puberty. β-Actin served as a loading control (A). Accordingly, time course of 20α-HSD enzymatic activity in female and male mice (n > 3 each) resembles the results from the expression analysis. Interestingly, decrease of 20α-HSD activity in male animals precedes the postpubertal increase of testosterone blood levels (B). Immunohistochemistry demonstrates 20α-HSD expression exclusively in the X-zone, which lacks 3β-HSD expression. Accordingly, X-zone regression in the postpubertal male mouse is accompanied by loss of 20α-HSD staining (C). *Bars* in C represent 500 and 100 μm, respectively. D, Definitive zone; H&E, hematoxylin and eosin; M, medulla; X, X-zone.

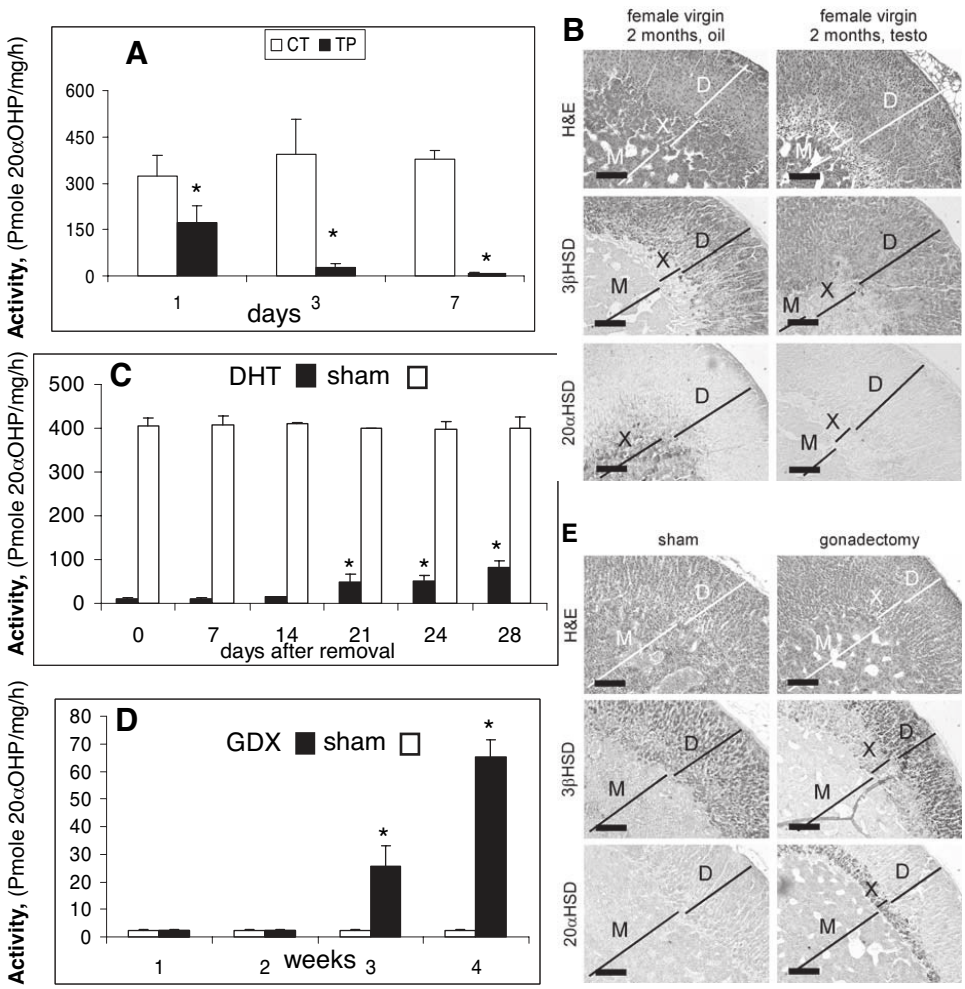

FIG. 2. Androgens suppress adrenal 20α-HSD activity *in vivo*. Testosterone propionate (TP) treatment in virgin female mice (n = 4 each) results in a rapid inhibition of adrenal 20α-HSD enzymatic activity within 7 d after treatment, and protein expression measured by Western blotting showed a similar inhibition (data not shown). *, Significant difference from the control (CT) group (A), which is accompanied by X-zone regression and loss of 20α-HSD-expressing cells (B). Conversely, partial regeneration of 20α-HSD activity is evident after removal of transplanted DHT-containing Silastic capsules in comparison with sham-treated controls. *, Significant difference from the 0-d activity (C). Conversely, gonadectomy (GDX) in postpubertal male animals (n > 7 each) is followed by an increase in 20α-HSD activity. *, Significant difference from the sham (D) and restoration of the 20α-HSD expressing X-zone, 4 wk post gonadectomy (E). *Bars* in B and E represent 500 μm. D, Definitive zone; H&E, hematoxylin and eosin; M, medulla; X, X-zone.

affect enzymatic activity (404.7 ± 18.4 pmol 20α-OHP/μg·h at d 0 and 400.8 ± 24.5 pmol 20α-OHP/μg·h at d 28, $P = 0.9$). Similar results could be obtained with testosterone implants, which resulted in a drop of adrenal 20α-HSD activity within 7 d (baseline 343.8 ± 20.6 pmol 20α-OHP/μg·h to 0.6 ± 0.5 pmol 20α-OHP/μg·h). In these experiments, serum testosterone levels in treated animals (11.3 ± 0.8 ng/ml) were well within the physiological range of those of postpubertal male animals (12.3 ± 2.1 ng/ml; Fig. 1B), indicating that no pharmacological levels of testosterone are needed to provoke an effect on X-zone degeneration. On the contrary, surgical gonadectomy in postpubertal male mice was followed by an increase in 20α-HSD activity (2.3 ± 0.5 pmol 20α-OHP/μg·h after 1 wk and 65.4 ± 2.3 pmol 20α-OHP/μg·h after 4 wk, $P < 0.0001$; Fig. 2D), whereas sham surgery did not increase enzymatic activity above baseline (2.3 ± 0.5 pmol 20α-OHP/μg·h after 1 wk and 2.3 ± 0.5 pmol 20α-OHP/μg·h after 4 wk). On a morphological level, gonadectomy but not sham surgery was followed by X-zone regrowth with reappearance of 20α-HSD-expressing cells (Fig. 2E).

Taken together, testosterone and DHT treatment suppresses adrenal 20α-HSD activity *in vivo* and leads to X-zone regression, whereas withdrawal of the androgenic block is accompanied by growth of a secondary X-zone and reappearance of 20α-HSD expression.

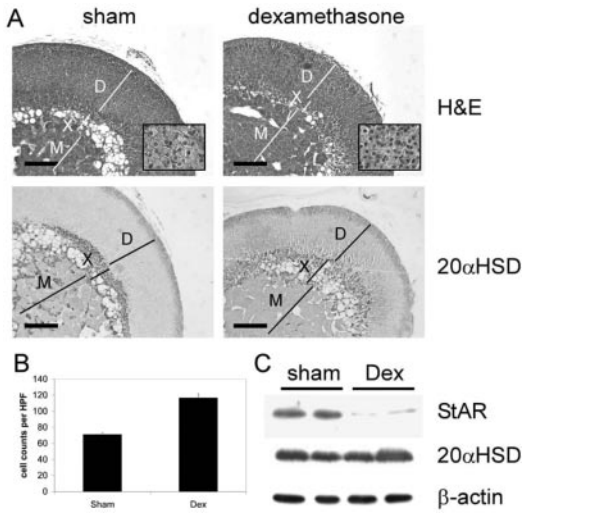

FIG. 3. Dexamethasone treatment does not affect X-zone growth or 20α-HSD expression. Although dexamethasone (Dex) treatment results in cellular hypotrophy with an increase in cell number per HPF (A and B) and decrease in the expression of StAR (C), it does not affect 20α-HSD staining (A) and expression pattern (C) in comparison with sham-treated controls (n = 3 per group). *Bars* in A represent 100 μm. D, Definitive zone; H&E, hematoxylin and eosin; M, medulla; X, X-zone.

Dexamethasone treatment does not affect X-zone regression or adrenal 20α-HSD expression

To further define possible effects of other steroids on adrenal 20α-HSD expression, female mice were treated with dexamethasone and compared with sham-treated age- and gender-matched control animals. As expected, short-time treatment with high doses of dexamethasone resulted in a significant decrease in adrenal weight (2.2 ± 0.1 mg *vs.* sham 3.0 ± 0.2 mg, $P = 0.05$), cellular hypotrophy within the zona fasciculata indicated by an decrease in cortical areas (610,453 ± 26,144 pixels *vs.* sham 806,857 ± 30,867 pixels, $P = 0.03$), and increase in the cell number per high-power field (HPF) (124.9 ± 7.0 cells per HPF *vs.* sham 77.5 ± 5.3 cells per HPF, $P < 0.0001$; Fig. 3, A and B). However, morphological characterization revealed no effect on X-zone regression or loss of 20α-HSD-expressing cells (Fig. 3A). Accordingly, although expression of StAR was down-regulated by dexamethasone treatment, adrenal 20α-HSD expression was not affected in dexamethasone-treated animals (Fig. 3C). Taken together, these data indicate that dexamethasone has no direct effect on X-zone growth. Moreover, in contrast to rapid effects on the morphology and steroidogenic profile of the zona fasciculata, 20α-HSD expression seems not to be affected by an ACTH-suppressive treatment of dexamethasone.

Pregnancy but not pseudopregnancy is accompanied by rapid loss of adrenal 20α-HSD activity and X-zone regression

As has been demonstrated earlier (3), ovarian 20α-HSD activity is down-regulated during pregnancy with a restoration of enzyme activity on the day before parturition to catabolize progesterone produced by the ovaries. When assayed in nulliparous female mice, the ability of adrenal lysates to reduce progesterone to 20α-OHP was within a similar range of activity (228.8 ± 36.8 pmol 20α-OHP/μg·h; n = 6) compared with lysates of ovaries from the same animals

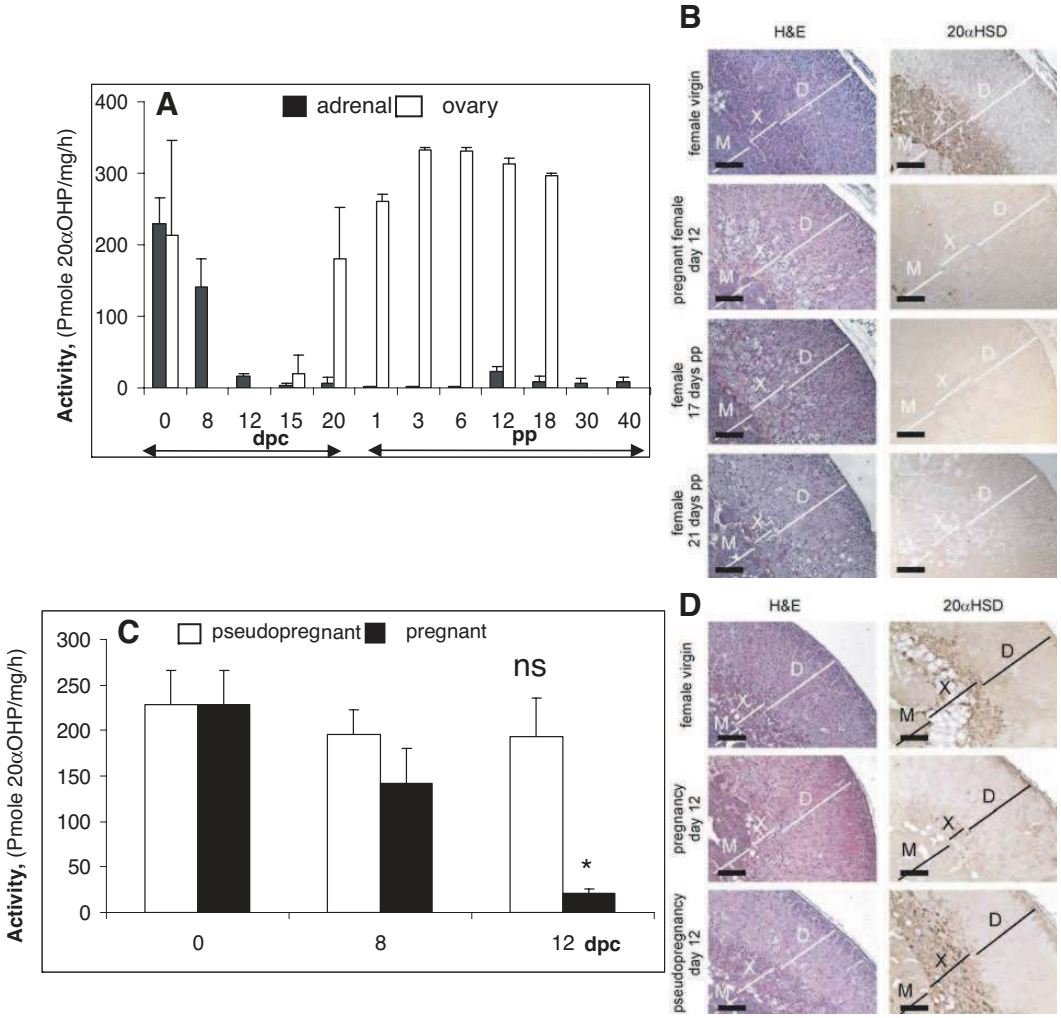

FIG. 4. The effects of pregnancy and pseudopregnancy on adrenal and ovarian 20α-HSD expression and activity. 20α-HSD enzymatic activities of adrenals and ovaries were determined in female mice (n = 6–8 mice per time point) during pregnancy and postpartum (pp) with 20 dpc being the last day of pregnancy. This time course during and after pregnancy demonstrates loss of adrenal 20α-HSD activity (A), which is paralleled by X-zone regression (B) with the development of lipid vacuoles (24). Although adrenal 20α-HSD activity remains low after pregnancy, ovarian 20α-HSD activity increases during parturition and postpartum (A). In contrast, pseudopregnancy does not affect adrenal 20α-HSD activity. *, Significant; ns, not significant, difference from the levels on d 0 (C) or immunohistochemical staining (D) (n = 3–5 mice per time point). *Bars* in B and D represent 100 μm. D, Definitive zone; H&E, hematoxylin and eosin; M, medulla; X, X-zone.

(213.4 ± 132.6 pmol 20α-OHP/μg·h; n = 5; Fig. 4A). Thus, it was of interest to follow the pattern of adrenal 20α-HSD activity during pregnancy. Indeed, similar to the ovarian enzymatic activity, the adrenal activity of 20α-HSD decreased during pregnancy to 10% at 12 dpc (16.6 ± 3.8 pmol 20α-OHP/μg·h) and completely disappeared thereafter. However, in contrast to the activation of the ovarian enzyme before parturition at 20 dpc (180.5 ± 72.5 pmol 20α-OHP/μg·h), adrenal activity was not restored, and it remained low even 40 d post parturition (7.6 ± 7.2 pmol 20α-OHP/μg·h; Fig. 4A). This time course of adrenal 20α-HSD activity was mirrored by a rapid X-zone regression and loss of 20α-HSD-positive X-zone cells after immunohistochemical staining (Fig. 4B). The appearance of a secondary X-zone containing lipid vacuoles in postpartum females is probably age related and has been reported before (24).

In contrast, induction of pseudopregnancy had no effect on 20α-HSD activity (pregnancy d 12, 21.4 ± 4.8 pmol 20α-OHP/μg·h, *vs.* d 0, 228.8 ± 36.8 pmol 20α-OHP/μg·h; $P < 0.01$; pseudopregnancy d 12, 193.4 ± 42.0 pmol 20α-OHP/μg·h, *vs.* d 0, 228.8 ± 36.8 pmol 20α-OHP/μg/h; $P = 0.63$; Fig. 4C) or X-zone regression (Fig. 4 D). Although the progesterone serum levels in pseudopregnancy are comparable to those in pregnancy within the first 8 d after conception, on d 12, pseudopregnancy is terminated as reflected by the decrease in blood progesterone levels to normal (nonpregnant) levels (data not shown). These findings indicate that the hormonal environment shared by the pregnant and pseudopregnant mice (*e.g.* prolactin secretion and levels of estrogen and progesterone) for at least 8 d are not sufficient to induce X-zone regression and decrease of adrenal 20α-HSD enzymatic activity.

PGF2α has distinct effects on ovarian and adrenal 20α-HSD activity

Because PGF2α induces 20α-HSD expression in the corpus luteum of pregnancy (3, 25), we tested its effect on adrenal enzyme activity. After two injections of 16-dpc pregnant mice with Estrumate (cloprostenol, synthetic PGF2α) at 4-h intervals, ovarian and adrenal 20α-HSD enzymatic activity was determined. As demonstrated previously, 20 h after the second injection, cloprostenol induced up-regulation of ovarian 20α-HSD enzymatic activity (66.3 ± 11.1 pmol 20αOHP/μg·h *vs.* 15.3 ± 3.6 pmol 20α-OHP/μg·h, $P < 0.001$; Fig. 5), which resulted in a significant reduction in progesterone serum concentration (55.1 ± 7.7 *vs.* 14.8 ± 8.0 ng/ml; $P < 0.001$) and premature parturition. In contrast, Estrumate had no significant effect on adrenal 20α-HSD activity, which remained low (3.1 ± 2.5 *vs.* 1.3 ± 0.5 pmol 20α-OHP/μg·h; $P = 0.16$, Fig. 6). Taken together, these findings underline the differences in prostaglandin-dependent regulation of ovarian and adrenal 20α-HSD activity.

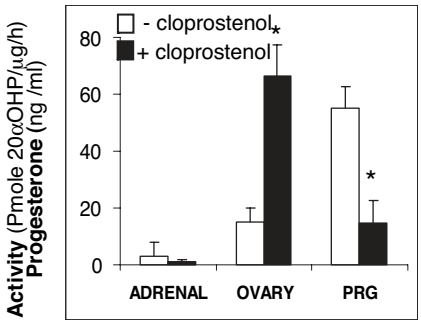

FIG. 5. Estrumate (cloprostenol) has diverse effects on ovarian and adrenal 20α-HSD activity at late pregnancy. Pregnant mice at 16 dpc (n = 4–6) were injected with either cloprostenol or PBS and killed 20 h later. As expected, cloprostenol treatment is followed by an induction of ovarian 20α-HSD activity and reduction in serum progesterone levels. In contrast, cloprostenol does not affect adrenal 20α-HSD activity. The same y-axis scale is used for enzyme activity and progesterone (PRG) concentration. *, Significant difference from the sham.

Lactation inhibits 20α-HSD expression and enzyme activity, whereas prolactin supplementation or disruption of Stat5-dependent pathways has no effect on X-zone growth

Because prolactin is one of the peptide hormones secreted at high levels during pregnancy, we further evaluated the effects on 20α-HSD activity under other physiological conditions with high serum prolactin levels. As such, female mice after pregnancy were divided into lactating and nonlactating animals, and adrenal 20α-HSD activity was determined. Interestingly, although enzymatic activity remained low in nursing females (2.6 ± 0.5 pmol 20α-OHP/μg·h), removal of the pups led to a partial recovery of 20α-HSD activity, measured 30 d post parturition (28.0 ± 1.8 pmol 20α-OHP/μg·h; $P < 0.001$; Fig. 6A). Accordingly, nursing was accompanied by a retained loss of 20α-HSD-expressing cells, whereas animals after parturition without nursing displayed partial regrowth of 20α-HSD-expressing X-zone cells (Fig. 6B).

In contrast, injection of 100 μg prolactin in nulliparous female mice twice a day for 4 d did not significantly affect adrenal 20α-HSD activity (527 ± 65 pmol 20αOHP/μg·h) in comparison with sham-treated animals (444 ± 77 pmol 20αOHP/μg·h, $P = 0.52$; Fig. 6C). Moreover, disruption of Stat5-dependent pathways, which are activated by prolactin, did not affect X-zone growth kinetics as evident in a normally regressed X-zone in postpubertal male mice (Fig. 6D, *upper* panel) and unaltered X-zone in virgin female mice (Fig. 6D, *lower* panel) with targeted disruption of Stat5a and -b.

Taken together, although suckling has a lasting effect on X-zone degeneration in primiparous female animals, prolactin is unlikely to be a major contributor to X-zone regression in other physiological instances.

Targeted deletion of 20α-HSD does not affect X-zone growth and regression

Because 20α-HSD expression affects local steroid metabolism, expression of the enzyme could result in paracrine effects required for adrenocortical growth and zonation. However, comparison of X-zone morphology between wild-type female animals and age- and sex-matched mice with targeted deletion of 20α-HSD (20α-HSD −/−) revealed no gross differences in adrenocortical morphology (Fig. 7A). Moreover, postpubertal X-zone regression and regrowth after gonadectomy in male animals was not affected in 20α-HSD −/− animals (Fig. 7B). Taken together, these data give evidence that loss of adrenal (and ovarian) 20α-HSD activity

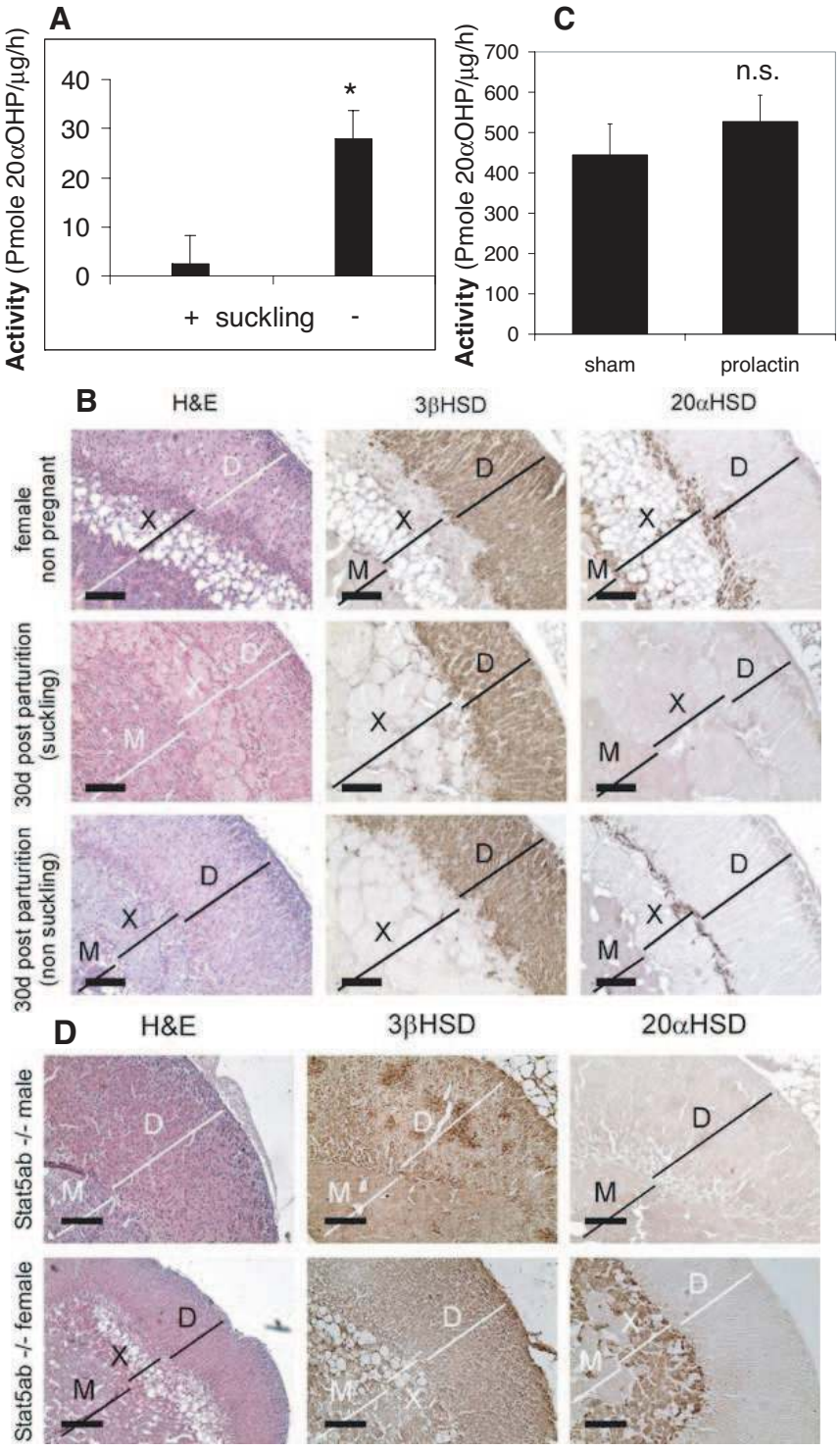

FIG. 6. Suckling maintains lack of adrenal 20α-HSD activity in postpartum female mice. In comparison with suckling mothers, postpartum nonsuckling animals display a significantly (*) higher level of adrenal 20α-HSD enzyme activity (n = 6–8) (A) and 20α-HSD expression by immunohistochemistry (B). Prolactin treatment was not associated with changes in adrenal 20α-HSD activity (n = 3–4 per group) (C). Moreover, disruption of Stat5-dependent pathways, which are activated by prolactin, did not affect X-zone growth kinetics as evident in a normally regressed X-zone in postpubertal male mice (D, *upper panel*) and unaltered X-zone in virgin female mice (D, *lower panel*) with targeted disruption of Stat5a and -b (Stat5ab −/−). *Bars* in B and D represent 500 and 100 μm, respectively. D, Definitive zone; H&E, hematoxylin and eosin; M, medulla; n.s., not significant; X, X-zone.

has no direct effect on adrenal morphology and X-zone growth dynamics.

Adrenal 20α-HSD reduces progesterone to 20α-OHP and DOC to 20α-OHDOC

To specify and extend the *in vivo* findings on adrenal 20α-HSD enzymatic activity, we generated recombinant mouse 20α-HSD to perform *in vitro* studies under well defined experimental conditions. In addition to progesterone, recombinant protein was incubated with DOC under reducing conditions (see *Materials and Methods*). After HPLC analysis, the percentage of formed 20α-OHDOC was calculated (Fig. 8B). As expected, the recombinant enzyme displayed enzymatic activity for the reduction of progesterone (138,900 ± 10,080 pmol 20α-OHP/μg·h; n = 4). In addition, reduction of DOC to 20α-OHDOC was readily detectable

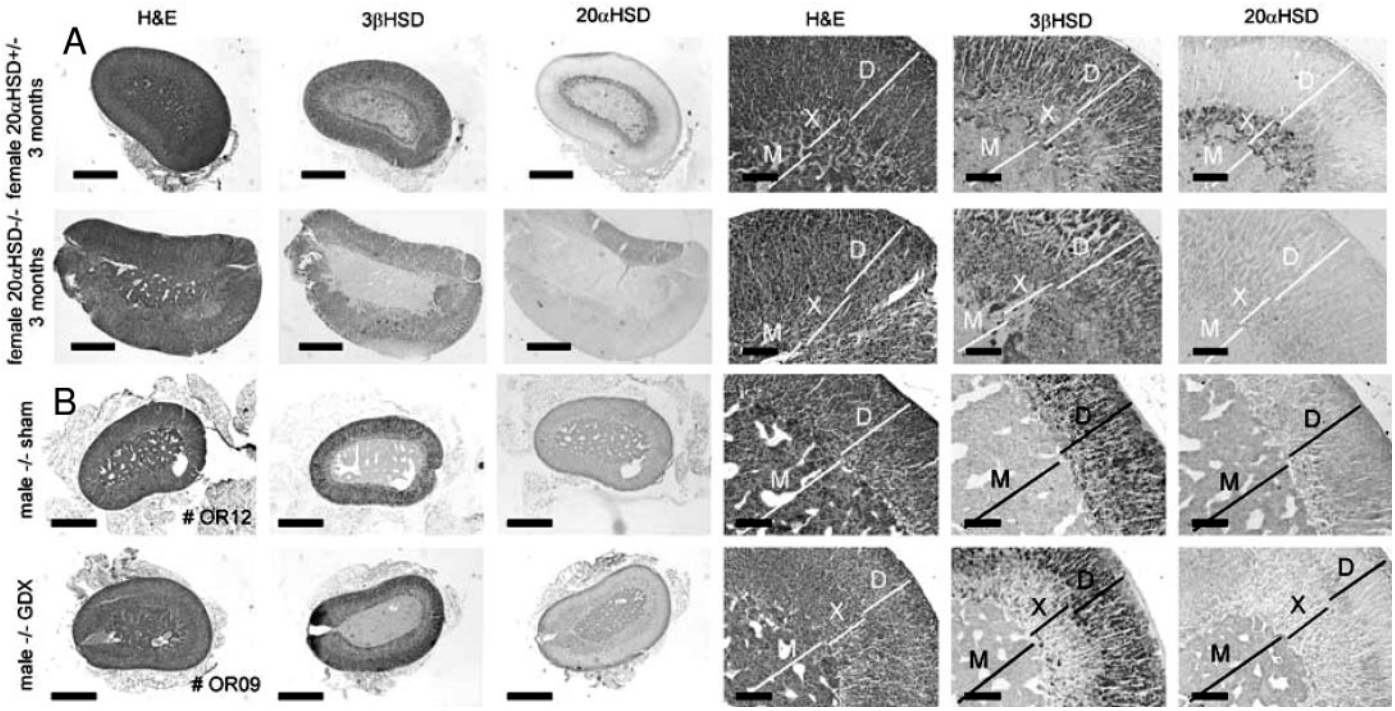

FIG. 7. Lack of 20α-HSD expression does not affect adrenal morphology or X-zone growth. Morphological examination of mice with targeted deletion of 20α-HSD (20α-HSD −/−) reveals no apparent differences in comparison with heterozygous controls (20α-HSD +/−) (A). Moreover, regression and regrowth of the X-zone in postpubertal and gonadectomized 20α-HSD −/− male animals, respectively, is not affected (B). *Bars* in A and B represent 500 and 100 μm, respectively. D, Definitive zone; H&E, hematoxylin and eosin; M, medulla; X, X-zone.

(31,390 ± 950 pmol 20α-OHDOC/μg·h; n = 3; Fig. 8B). Calculation of the kinetic parameters from conversion rates (Fig. 8D) revealed similar K_m for the reduction of progesterone (K_m = 5.80 μM) and DOC (K_m = 3.30 μM) but higher V_{max} when progesterone (V_{max} = 133.18 nmol/30 min·μg) was used as the substrate in comparison with DOC (V_{max} = 7.39 nmol/30 min·μg). Similarly, we demonstrated the ability of adrenal lysate from 20α-HSD +/− mice to reduce DOC to 20α-OHDOC (47.1 ± 2.9 pmol 20α-OHDOC/μg·h; n = 3), whereas the homogenates taken from 20α-HSD −/− animals displayed no measurable activity (0.0 ± 0.0 pmol 20α-OHDOC/μg·h; Fig. 8E). Taken together, these findings give good evidence for a role of murine 20α-HSD as a progesterone- and DOC-metabolizing enzyme within the adrenal gland. In contrast, no 3-keto reductase activity with progesterone or DOC as substrates and no corticosterone or PGF2α reductase activity was detected using the recombinant protein (data not shown).

Discussion

Herein we provide evidence that the X-zone of the murine adrenal cortex is specified by its expression of 20α-HSD. First, immunohistochemical staining revealed X-zone-specific expression of adrenal 20α-HSD. Second, in all experimental paradigms studied, adrenal 20α-HSD enzymatic activity was positively associated with the presence (or absence) of the adrenal X-zone. In accordance with this notion, time-course experiments reveal adrenal 20α-HSD expression in both male and female mice. However, the pattern of expression considerably differs between the genders; whereas 20α-HSD expression in the female adrenal is maintained over an extended period of time, expression in the male is restricted to a short time window before puberty around the third week of life. Expression of 20α-HSD and presence of enzymatic activity in the mouse adrenal have been reported previously. Interestingly, in these reports, 20α-HSD was detected only in females, whereas expression in the male adrenal was missed (8, 20, 21), most likely because of its temporally restricted expression.

Although a number of earlier studies have attempted to delineate its origin and function (11, 26), only recently have tracing experiments in animals with transgenic expression of lacZ under the control of a fetal adrenal-specific enhancer of the SF-1 gene (FAdE) demonstrated that the X-zone is a direct derivative of the fetal zone of the adrenal cortex (14). Although the fetal zone in primates plays a role in glucocorticoid and androgen homeostasis during pregnancy (27), the functional role of the X-zone has remained elusive despite its early morphological description and ultrastructural characterization indicating steroidogenic properties (28). Notably, we (data not shown) and others (21) did not detect 20α-HSD expression in the developing adrenal cortex. Although this does not contradict the published tracing experiments (14), the lack of adrenal 20α-HSD expression in the embryo together with the unaltered adrenal morphology in 20α-HSD knockout animals provide evidence that 20α-HSD activity is not required for proper adrenal development.

Early studies have revealed that castration prevents X-zone degeneration in male mice (11) and castration after degeneration results in the formation of a secondary X-zone

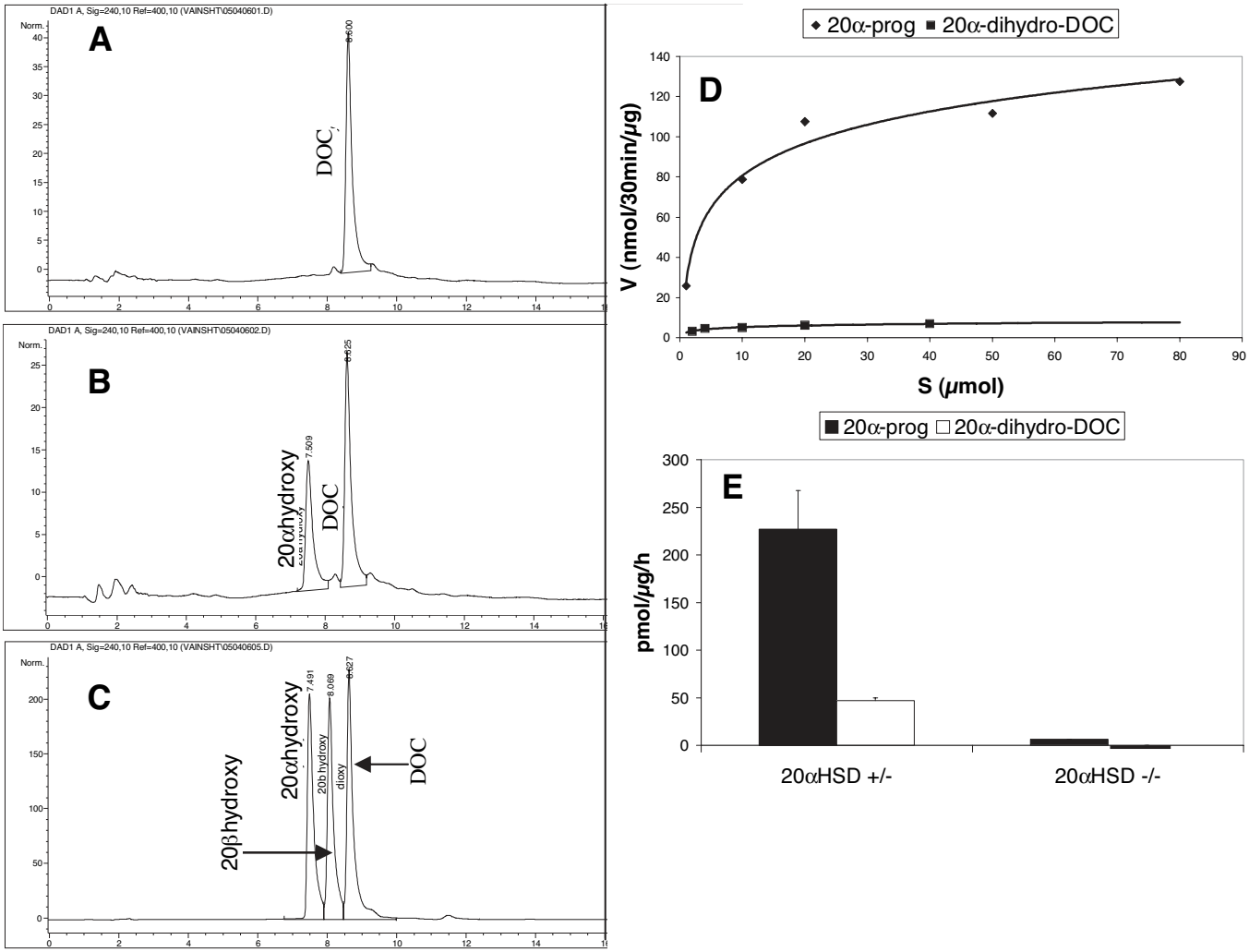

FIG. 8. Murine 20α-HSD reduces progesterone and DOC. After incubation of recombinant mouse 20α-HSD with DOC, the substrate and metabolites were extracted and analyzed by HPLC as described in *Materials and Methods*. HPLC traces are shown: reaction mixture without the enzyme (A), reaction mixture including the recombinant enzyme (B), and standards of DOC, 20β-OHDOC, and 20α-OHDOC (C). Note adjusted y-axis scale in the individual HPLC traces. Quantification of enzyme activity for the reduction of progesterone and DOC reveals similar K_m for the reduction of progesterone (K_m = 5.80 μM) and DOC (K_m = 3.30 μM) but higher V_{max} for progesterone (V_{max} = 133.18 nmol/30 min·μg) in comparison with DOC (V_{max} = 7.39 nmol/30 min·μg) (D). Enzymatic activity of endogenous 20α-HSD with DOC or progesterone as substrates is detectable only in adrenal homogenates from heterozygous 20α-HSD knockout control mice (20α-HSD +/−), whereas adrenals from homozygous 20α-HSD knockout animals (20α-HSD −/−) display no or only very low steroid turnover (E).

(29). These studies led to the hypothesis that androgens are required for the degeneration of the X-zone. Indeed, testosterone treatment results in fast X-zone regression and suppression of adrenal 20α-HSD activity, whereas withdrawal of androgens by gonadectomy is followed by partial restoration of adrenal 20α-HSD expression. However, the X-zone still degenerates in *tfm* mice, which have a defect in the androgen receptor gene (30), suggesting that the degeneration process does not necessarily require androgen binding to the androgen receptor in the adrenal cortex or other tissues (30). Sequence analysis of the murine 20α-HSD promoter region (3 kb upstream from the coding region) revealed potential binding sites for glucocorticoid receptor and additional, different, binding sites for progesterone receptor as well as potential cAMP response element binding protein binding site, whereas no androgen receptor binding sites have been predicted (31). It is possible that the androgen might be modified (*e.g.* by sulfation) or converted to another steroid that in turn acts as the active substance that induces X-zone degeneration. However, as the observed drop of adrenal 20α-HSD activity in male mice precedes the pubertal increase in serum testosterone, collectively, these data suggest that testosterone itself is probably not the sole mediator of transcriptional regulation of 20α-HSD expression or of X-zone regression.

Other hormones such as activin and inhibin (32) and nuclear transcription factors such as Dax-1 (16) have been demonstrated to play a role in X-zone regression, and the specific role of these factors for puberty-associated X-zone regression needs to be determined. In addition, the induced stimulation and inhibition of pituitary gonadotropin secretion by gonadectomy and androgen treatment, respectively, is likely to affect the adrenal phenotype including adrenal 20α-HSD expression *in vivo*. Specifically, induction of LH receptor expression in the murine adrenal cortex by gonadectomy has

been demonstrated by us (32) and others (33). In fact, there is an increasing body of evidence suggesting that under these experimental conditions, LH can act as a growth factor for the adrenal cortex (32, 34); because gonadotropin receptors are not expressed in the mouse adrenal at baseline conditions, the fast effects of androgen treatment on X-zone regression are unlikely to be caused by suppression of pituitary LH and FSH.

Adrenal 20α-HSD activity is regulated during and after pregnancy: The well described involution of the X-zone within the first pregnancy (12, 35) is associated with the complete loss of adrenal 20α-HSD activity. Because functional characterization of adrenal-derived 20α-HSD demonstrated enzymatic properties identical to that of the ovary, adrenal 20α-HSD activity might interfere with progesterone homeostasis during early pregnancy, whereas the parallel down-regulation of adrenal and ovarian 20α-HSD activity at later time points provides the basis for maintenance of high progesterone levels during midpregnancy. However, considering a functional role of the X-zone for pregnancy, undulating re-formation before and regression during concomitant pregnancies would be expected. It has been demonstrated earlier on a morphological basis that effects of pregnancy-induced X-zone regression seems to be permanent (36). Likewise, we were not able to detect reappearance of adrenal 20α-HSD activity during long-term follow-up (30 and 40 d) of female animals after their initial pregnancy (data not shown). Moreover, 20α-HSD −/− mice have no difficulties becoming pregnant. Thus, adrenal 20α-HSD expression seems not to be required for concomitant pregnancies in the mouse.

Although no changes in adrenal 20α-HSD activity or X-zone morphology were evident in either pregnant or pseudopregnant animals at early time points, 20α-HSD activity diminished from 12 dpc onward in parallel with the regression of the X-zone in pregnant but not pseudopregnant animals. In rodents, vaginal nerve stimulation during copulation results in prolactin surges, which results in enhanced progesterone synthesis of the corpus luteum (37, 38). Upon d 10 of pregnancy, pituitary prolactin surges cease and the constitutive secretion of placental lactogens replaces the prolactin function as luteotropin (39, 40). Our findings on pseudopregnant animals support the hypothesis that during the first half of pregnancy, the daily surges of pituitary prolactin are not sufficient to induce X-zone regression and down-regulation of adrenal 20α-HSD activity, whereas the constitutive secretion of placental lactogens might be required to block adrenal 20α-HSD activity. Interestingly, postpartum regeneration of the X-zone that occurred in mothers that were restrained from nursing their pups indicates a possible involvement of prolactin also in postpartum X-zone plasticity.

To further delineate the potential role of prolactin on adrenal 20α-HSD expression and X-zone growth, we administered prolactin to nulliparous female mice. This short-term treatment, however, did not affect adrenal 20α-HSD enzyme activity. Because prolactin acts through activation of the Jak/Stat pathway, targeted deletion of Stat5a/b disrupts the prolactin-dependent signaling cascades (22). In this context, Stat5a/b knockout animals have been used as a model to dissect prolactin-dependent effects on the ovary (3). As demonstrated, disruption of the Stat5-dependent pathway has no profound effect on X-zone morphology and 20α-HSD expression. Unfortunately, because female Stat5a/b knockout animals are not fertile (3), we could not evaluate pregnancy and/or lactation-related X-zone regression in this model. Moreover, we cannot exclude the possibility that prolactin-dependent effects on X-zone growth kinetics might be mediated through Stat5-independent mechanisms (41, 42). In addition to prolactin, suckling stimulates secretion of oxytocin from the pituitary (43, 44). Oxytocin has been reported to affect the hypothalamo-pituitary-adrenal axis (45) as well as adrenal steroidogenesis (46). Thus, additional oxytocin-dependent mechanisms (alone or in concert with prolactin) have to be taken into account for X-zone-related effects during lactation.

Taken together, it is possible that prolactin might have a direct effect on adrenal 20α-HSD expression and X-zone regression. However, because the applied experimental paradigms all have their specific limitations, the physiological significance of these findings has to be addressed in more detail in future studies.

At late pregnancy, the induction of parturition involves the PGF2α-dependent up-regulation of ovarian 20α-HSD, which results in a drop of progesterone levels (4, 47). Accordingly, cloprostenol (synthetic PGF2α) triggers the expression of ovarian 20α-HSD in pregnant mice (3, 48). In contrast, cloprostenol treatment did not affect adrenal 20α-HSD activity in the same animal. The apparent difference in prostaglandin-dependent up-regulation of 20α-HSD in the ovary and the adrenal cortex is most likely to be explained by the lack of 20α-HSD-expressing cells after X-zone regression that cannot be acutely replaced through growth regulation.

Acute adaptation of adrenal steroidogenesis to the demands of the organism is regulated on a functional level through transcriptional activation of steroidogenic enzymes. However, structural plasticity of the adrenal cortex dictated by cellular proliferation, differentiation, and apoptosis is crucial for long-term adrenal adaptation. In humans, postpartum regression of the fetal zone and restoration of adrenal androgen production after formation of the zona reticularis during adrenarche are examples of the overall impact of this functional imprinting of adrenal zonation on steroid metabolism. Moreover, the recently proposed alternative pathway in human adrenal androgen synthesis that is dependent on the presence of 5α-reductase and 3α-HSD in the fetal zone of the adrenal cortex provides additional insights into potential important pathways that are regulated mainly by zonation of the adrenal cortex (49). However, although our observations suggest that adrenal 20α-HSD expression is dependent on the presence of a vital X-zone, we cannot exclude additional regulatory mechanisms taking place on the transcriptional and posttranscriptional level.

20α-HSD has been initially identified in rodents as a progesterone-catabolizing enzyme of the ovary (1), which has later been shown to be actively involved in the control of progesterone homeostasis during pregnancy (3). Herein, we provide evidence suggesting that murine 20α-HSD is also able to reduce DOC to 20α-OHDOC albeit with a lower enzymatic activity. In contrast, no 3-keto-reductase activity

of recombinant 20α-HSD was evident with progesterone or DOC as substrates, and no corticosterone or PGF2α reductase activity was detectable (data not shown). A similar degree of specificity has been obtained for the highly homologous rat 20α-HSD enzyme, which is specific for the modification of the C20-keto position and reduces progesterone and 17-OHP but not corticosterone and has no detectable 3α- or 17β-HSD or prostaglandin-reducing activity (50, 51). In humans, at least four aldo keto reductases (AKR1C1, AKR1C2, AKR1C3, and AKR1C4) have been shown to possess 20α-HSD activity for progesterone at varying degrees. In contrast to the rodent homologs, however, these enzymes exhibit broader enzymatic properties including 3α- and 17β-HSD activity (52). Similar to our findings for the murine enzyme, it has been reported that AKR1C3 reduced DOC to 20α-OHDOC whereas AKR1C1, AKR1C2, and AKR1C4 are devoid of such properties (53).

Because 20α-HSD expression affects local steroid metabolism in the adrenal gland, expression of the enzyme is likely to modulate the paracrine milieu within the adrenal cortex. Furthermore, it is conceivable that 20α-HSD might be involved in the metabolism of other substrates such as sterols that could be involved in the regulation of adrenocortical zonation. However, because morphological characterization of adrenals from 20α-HSD −/− animals revealed no apparent effect on growth or regression of the X-zone in the absence of 20α-HSD activity, 20α-HSD expression and function itself is unlikely to play a major role in the regulation of adrenal growth dynamics.

Because DOC is an intermediate in the biosynthesis of corticosterone and aldosterone, murine 20α-HSD may, in addition to its ability for metabolizing progesterone, also be involved in the control of glucocorticoid and mineralocorticoid homeostasis. Human AKR1C3 is expressed in the mineralocorticoid-responsive epithelial cells of the renal cortical and medullary collecting ducts and in the colon. Thus, it has been suggested that AKR1C3 might act as a safeguard for the mineralocorticoid receptor to prevent activation by DOC in the kidney and colon (53). Similarly, the expression pattern of murine 20α-HSD in nonsteroidogenic tissues, including the kidney and hematopoietic cells (3), gives indirect evidence for a potential involvement of 20α-HSD in the modulation of steroid action in these target tissues. Thus, although DOC is not synthesized in the X-zone as suggested by the absence of 3β-HSD immunoreactivity, 20α-HSD could act by inactivating DOC that is secreted from the adjacent adrenal cortex. However, the true physiological function of adrenal 20α-HSD is not yet clear.

Taken together, we demonstrate that the murine X-zone is defined by its restricted expression profile for 20α-HSD. We further provide evidence indicating that in addition to progesterone, DOC can serve as a substrate for murine 20α-HSD. Although the physiological function of the X-zone for adrenal steroidogenesis is not yet clearly defined, our findings provide the basis for a role of the X-zone in modulation of progesterone and potentially glucocorticoid and mineralocorticoid metabolism. Furthermore, the presented data define 20α-HSD as a useful marker for future studies of the murine X-zone.

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