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Male Marmoset Monkeys Express an Adrenal Fetal Zone at Birth, But Not a Zona Reticularis in Adulthood

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Neonatal human males produce high levels of dehydroepiandrosterone (DHEA) and its sulfo-conjugated form (DS) that decline within a few months of birth, due to regression of the adrenal fetal zone (FZ). Adult male humans and rhesus monkeys produce C19 steroids in abundance from the adrenal zona reticularis (ZR). Male marmoset monkeys produce DS at birth, but unlike humans and rhesus monkeys, do not produce comparable amounts of DHEA and DS in adulthood. To determine whether male marmosets express a functional ZR in adulthood, we examined adult and neonatal male marmosets for the presence of a ZR and FZ, respectively. Exogenous ACTH failed to stimulate DHEA or DS in adults, and dexamethasone treatment failed to suppress DHEA and DS, although cortisol levels changed as expected. In steroidogenic tissues, the key proteins necessary to synthesize C19 steroids from pregnenolone are P450c17, 3β-hydroxysteroid dehydrogenase (3β-HSD), nicotinamide adenine dinucleotide phosphate (reduced) oxido-reductase cytochrome P450 (reductase), and cytochrome$_{b5}$ (cyt$_{b5}$). Adult adrenal cross sections showed P450c17 and reductase protein expression throughout the cortex but showed no expected decrease in 3β-HSD and increase in cyt$_{b5}$ in the innermost region. Western analysis confirmed these data, demonstrating comparable P450c17 expression to rhesus monkeys, but not cyt$_{b5}$. HPLC analysis revealed similar 17α-hydroxylase action on pregnenolone for adult marmoset and rhesus adrenal microsomes but greatly diminished 17,20-lyase activity in marmosets. Neonatal marmoset adrenals exhibited staining indicative of a putative FZ (with P450c17, reduced 3β-HSD and increased cyt$_{b5}$). We conclude that neonatal marmosets exhibit a C19 steroid-secreting FZ similar to humans, but adult males fail to acquire a functional ZR. (*Endocrinology* 146: 365–374, 2005)

THE COMMON MARMOSET (*Callithrix jacchus*) is a New World monkey that is widely used in biomedical research (1–3), including studies on stress (4) and the effects of behavioral subordination on adrenocortical function (5–7). Nonetheless, little is known of the structure or function of the fetal or adult marmoset adrenal and how it may differ from humans. Although adrenal morphology is relatively straightforward in other primate models, the histological zonation of the marmoset adrenal cortex has endured much controversy in the literature. Miraglia and Moreira (8) reported in 1969 that previous histological staining provided no conclusive evidence for the presence or absence of a zona reticularis (ZR) in either the male or the female marmoset adrenal: the putative ZR was indistinct and difficult to describe definitively. There was, however, clear morphological evidence for the presence of a zona glomerulosa (ZG) and a zona fasciculata (ZF). In their study, Miraglia and Moreira (8) interpreted morphological evidence in favor of a ZR, although it was difficult to visualize and staining techniques were nonimmunospecific. Levine *et al.* (9) also performed hematoxylin and eosin staining of marmoset adrenals and found evidence of a fetal zone (FZ) in neonates, but no expression of a ZR in adult males or females. The adults lacked morphologically distinct cell types between the mid-adrenal and the innermost adrenocortical region (the cells abutting the cortico-medullary junction). The finding of Levine *et al.* (9) was substantiated by an extensive measurement of circulating steroids *in vivo*. Both male and female marmosets had relatively high circulating levels of dehydroepiandrosterone (DHEA; males 1343 ± 266 ng/dl, females 3110 ± 1083 ng/dl; mean ± SEM), and the sulfoconjugate form DS at birth that declined within a few months, consistent with the regression of a functional FZ (9). In contrast to humans and other primates, such as rhesus monkeys, however, male marmosets do not produce large amounts of DHEA and DS upon reaching sexual maturity (9). Thus, the question remains: is the lack of DS production due to an endocrine suppression mechanism or to lack of a functional ZR in the adrenal cortex of adult male marmosets?

Since the aforementioned studies were performed, there has been considerable progress in our understanding of the enzymology of adrenal steroidogenesis and the factors that underlie normal ZR function. The adrenal cortex is known to be the source of mineralocorticoids, glucocorticoids, and, in both human and many nonhuman primates, C19 steroids. The ability to synthesize these three classes of steroids is

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Abbreviations: cyt$_{b5}$, Cytochrome$_{b5}$; P450c17, 17α-hydroxylase/17,20-lyase cytochrome P450; CV, coefficient of variation; DHEA, dehydroepiandrosterone; DS, DHEA's sulfo-conjugated form; FZ, adrenal fetal zone; 3β-HSD, 3β-hydroxysteroid dehydrogenase; IHC, immunohistochemistry; ZF, zona fasciculata; ZR, zona reticularis.

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achieved through discrete adrenal zonation (Ref. 10 and Fig. 1). Mechanisms underlying zonation have been studied extensively in experimental models such as rat, mouse, and cow, and these have established that zonal function derives from the presence or, just as importantly, the absence of certain enzymes. Although these nonprimate models lack any significant ZR, studies in humans and nonhuman primates have demonstrated that the adult ZR is capable of producing C19 steroids, most specifically DHEA and DS, due to its high expression of 17α-hydroxylase/17,20-lyase cytochrome P450 (P450c17) combined with low expression of competing 3β-hydroxysteroid dehydrogenase (3β-HSD) (Refs. 11–16 and Fig. 1). In addition, recent studies suggest that the ZR is also often rich in cytochrome$_{b5}$ (cyt$_{b5}$) (14–16).

Despite these advances in our understanding of ZR function, immunospecific staining for key steroidogenic enzymes in the adrenal, and correlative *in vivo* challenges of adrenocortical function, have not been performed on male marmosets. Our aim, therefore, was to localize the enzymes and cofactors influencing DHEA production to specific adrenal zones and to correlate those findings with results of functional manipulation of circulating levels of adrenal steroids. We found that although male marmosets exhibit a neonatal adrenal morphology and steroid production pattern that is developmentally similar to humans, they do not express a functional ZR upon maturation to adulthood. These unique properties make the male marmoset a highly unusual model that shares developmental similarities to humans, but in adulthood, is sufficiently different that it would be of considerable value in determining what factors may be necessary for normal human ZR development and function.

Materials and Methods

Materials

Unless otherwise stated, general reagents were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific International (Pittsburgh, PA).

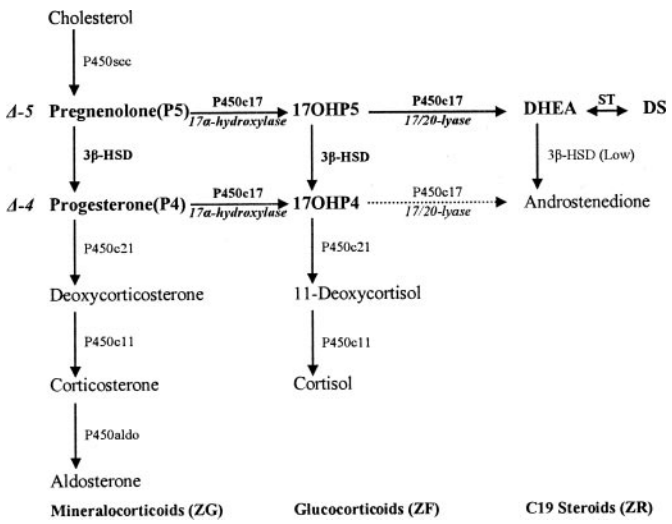

FIG. 1. Steroid biosynthesis in the adrenal cortex of higher mammals. *Solid arrows*, Predominant pathway in species expressing P450c17 in the adrenal gland. *Dashed arrows*, Steroid biosynthesis seen in species using the Δ-4-lyase pathway.

Animals

This research was conducted in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act and its subsequent amendments. All animal procedures were reviewed and approved by the Graduate School Animal Care and Use Committee of the University of Wisconsin-Madison. The National Primate Research Center at the University of Wisconsin-Madison (WPRC) is accredited by AAALAC as part of the UW-Madison Graduate School. Adult male marmosets, as detailed below, were housed indoors at the WPRC. They lived in aluminum and welded wire cages measuring 61 × 91 × 183 cm or 122 × 61 × 183 cm, or in a large room measuring 363 × 212 × 218 cm. Males were housed either singly, with an adult female pairmate or in a larger mixed-sex group, and had visual, olfactory, and auditory access to conspecifics in other cages. Lights were on from 0600–1800 h, and animals were fed at 1230–1330 h daily. Additional information on animal housing and husbandry has been published previously (6). Infant and adult rhesus monkey (*Macaca mulatta*) tissue was obtained, opportunistically, from healthy animals assigned as controls to other approved projects at the California National Primate Research Center.

Steroid responses in vivo

Adrenocortical responses to ACTH$_{1–39}$ (ACTH$_{1–39}$) were determined in four adult male marmosets, which were 38–62 months of age and weighed 365 ± 8 g (mean ± SEM) at the beginning of data collection. Four doses of human ACTH$_{1–39}$ (0.1, 1, 10, and 100 μg/kg) and a saline control were administered to each male at intervals of at least 2 wk, with the order of treatments balanced across animals. Males were weighed and injected with dexamethasone (5 mg/kg, im; American Regent Laboratories, Shirley, NY) at 1700 h to suppress endogenous ACTH secretion. The following morning at 0900 h, each male was administered human ACTH$_{1–39}$ or saline iv. Blood samples (0.2–0.6 ml) were collected immediately before, 60 min after, and 120 min after ACTH$_{1–39}$ injection. In a separate experiment, adrenocortical responses to dexamethasone were determined in six adult male marmosets (25–54 months of age, 377 ± 11 g). Dexamethasone (5 mg/kg) was injected im at 1600 h, and blood samples (1.2 ml) were collected at 0900 h the day of and the day after dexamethasone treatment, within 3 min of initial disturbance to the animal. In both experiments, marmosets were briefly restrained in a marmoset restraint tube (17), whereas blood was collected from the femoral vein using a heparinized 1-cc syringe and 27-gauge needle. Samples were immediately placed on ice and centrifuged at 2000 rpm for 10 min, and the plasma fractions were aspirated and frozen at −20 C until assayed.

Hormone assays

All plasma hormone concentrations were determined by RIAs that had been validated for use with marmoset plasma at the WPRC Assay Services laboratories. The cortisol and testosterone assays were described previously (5, 18). Their intra- and interassay coefficients of variation (CVs) were 5.65% and 10.48%, respectively, for cortisol, and 2.2% and 5.5%, respectively, for testosterone. The remaining RIAs for corticosterone (antibody: Esoterix Endocrinology, Calabasas Hills, CA; tritiated trace: American Radiolabeled Chemicals, Inc., St. Louis, MO; reference preparation: Sigma-Aldrich), DHEA (antibody-coated tube RIA, TKDH2; DPC, Los Angeles, CA), DS (antibody-coated tube RIA, TKDS1; DPC) and aldosterone (antibody-coated-tube RIA, Active Aldosterone; Diagnostics Systems Laboratories, Inc., Webster, TX), produced the following intra- and interassay CVs: corticosterone, 2.2% and 9.0%; DHEA, 3.6% and 4.2%; DS, 1.7%, interassay CV not available for a single assay; and aldosterone, 2.3% and 5.4%. Corticosterone, testosterone, and DHEA RIAs were performed after celite chromatography.

The recovery of DS standards added to 50 μl of a marmoset plasma pool (n = 5) was 103.3 ± 2.2%. Serial dilution of a DS-spiked marmoset pool (50.00–0.39 μl; n = 8) gave a displacement curve parallel to that obtained with DS standards. The sensitivity of the assay was 5.0 μg/dl. The recovery of DHEA standards added to 100 μl of a marmoset plasma pool (n = 7) was 99.0 ± 2.4%. Serial dilution of a DHEA-spiked marmoset pool (100.00–1.56 μl; n = 7) gave a displacement curve parallel to that obtained with DHEA standards. The sensitivity of the assay was 0.5 ng/ml.

Immunohistochemistry (IHC)

Adrenals from adult male marmosets (aged 24–72 months, n = 6) were fixed in 10% formalin, processed routinely, and paraffin embedded. Adrenals from 1-d-old marmosets (n = 5 males, n = 1 female; 2 adrenals donated from the Laboratory of Behavioral Neurobiology, Swiss Federal Institute of Technology, and 4 from the WPRC) were fixed in 4% paraformaldehyde in 0.1 M sodium cacodylate buffer, processed routinely and embedded in paraffin wax. Tissues were then cut into 5-μm sections. All sections were deparaffinized and rehydrated in clearing solution and graded alcohols. Endogenous peroxidase activity was quenched by incubation in 30% hydrogen peroxide in 100% methanol for 30 min. Staining was detected using the Vectastain Elite ABC Kit (rabbit IgG) (Vector Laboratories, Inc., Burlingame, CA). Sections were subsequently stained using antiserum raised against human P450c17 (1:1000, chicken polyclonal, Dr. A. J. Conley, Department of Population Health and Reproduction, University of California-Davis), human placenta 3β-HSD (1:400, rabbit polyclonal, Dr. J. Ian Mason, Clinical Biochemistry, University of Edinburgh, Edinburgh, Scotland, UK), human cyt_{b5} (1:3000, rabbit polyclonal, Dr. A. J. Conley), rat nicotinamide adenine dinucleotide phosphate (reduced) oxido-reductase cytochrome P450 (reductase; 1:3000, rabbit polyclonal, Dr. A. J. Conley), human side-chain-cleavage cytochrome P450 (P450scc; 1:1000, rabbit polyclonal, Dr. W. L. Miller, Department of Pediatrics, University of California-San Francisco), and human 21-hydroyxlase cytochrome P450 (P450c21; 1:3000, rabbit polyclonal, Dr. W. L. Miller). Normal nonimmune rabbit serum (1:1000, Vector Laboratories, Inc.) was used as a control. All sections were incubated with diluted normal goat serum (1:200) for 20 min at room temperature to block nonspecific binding. They were then incubated with primary antibody diluted in buffer at 4 C overnight. The sections were incubated in diluted secondary antibody (1:200, antirabbit, or 5 μg/ml, antichicken) and avidin-biotin-conjugated peroxidase for 30 min each at room temperature before exposure to 3-amino-9-ethylcarbazole (Vector Laboratories, Inc.) for 10 min also at room temperature. Nuclei were counterstained blue with Gill 2 hematoxylin (neat, 2 sec, rinsed 5 min in tap water) before sections were coverslipped with Paramount (Dako Corp., Carpinteria, CA). For photo-quality purposes, some sections were restained using 3,3′-diaminobenzidine (Vector Laboratories, Inc.) for 5 min at room temperature, counterstained as above, dehydrated, and coverslipped with permanent mounting media. Images were captured using bright-field illumination on a Zeiss Axioskop (Carl Zeiss, Oberkochen, Germany) fitted with a SPOT2 digital camera (through Zeiss), and digital-grayscale densitometry (Zeiss software, KS 300 version 3.0) was then used to quantify relative staining.

Microsomal preparation and Western immunoblotting

Whole frozen adrenals from six adult male marmosets (aged 60–132 months) and two infant rhesus monkeys (1 male and 1 female, aged 3 and 4 months, respectively) were homogenized directly in lysis buffer [0.1 M KPO_4 (pH 7.4), 20% glycerol, 5 mM β-mercaptoethanol, and 0.5 mM phenylmethylsulfonyl fluoride] at a ratio of 0.1 g/1 ml tissue to buffer. Cellular debris was removed by centrifugation at 1000 $\times$ *g* for 10 min. The supernatant was then spun down at 16,000 $\times$ *g* for 10 min under the same conditions. The premicrosomal pellet was stored at −80 C, and the supernatant was spun down at 100,000 $\times$ *g* for 60 min. The pellet was rehomogenized in dialysis buffer [lysis buffer with 1 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate detergent] and stored at −80 C. Protein concentration of microsomal preparations was determined using a BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL). Ten (10) micrograms of total microsomal protein were size separated per lane on a 16% polyacrylamide gel (Bio-Rad Laboratories, Inc., Hercules, CA, 150 V constant, 1.5 h) and protein transferred to PVDF membrane (Bio-Rad Laboratories, Inc., 100 V, 1 h). Western blotting was then performed using the antihuman P450c17 and antihuman cyt_{b5} antibodies (P450c17 1:10,000, chicken horseradish peroxidase-linked IgY secondary antibody 1:5000, cyt_{b5} 1:2000, donkey antirabbit horseradish peroxidase-linked IgG secondary antibody 1:10,000, detected by ECL (Amersham Biosciences, Piscataway, NJ) as previously described (19). Recombinant human cyt_{b5} was used as a control.

P450c17 substrate preference and relative Δ-4-lyase deficiency assay/analysis

Synthesis of DHEA by marmoset and rhesus P450c17 of adrenal origin and relative Δ-4-lyase deficiency were examined by radio-HPLC analysis of substrate metabolism. Microsomal protein (20 μg) from the adrenals of six adult male marmosets (aged 60–132 months) and six juvenile and adult male rhesus monkeys (aged 3–96 months), prepared as above, were used in 0.5 ml assays. Pregnenolone (Steraloids, Newport, RI; [^{3}H]-pregnenolone, PerkinElmer, Boston, MA) was used as the substrate to examine DHEA synthesis capabilities of marmoset and rhesus adrenal P450c17. Treatment with and without the 3β-HSD inhibitor trilostane (50 μM; Sanofi Research Division, Great Valley, PA) was performed for each subject. Δ-4-Lyase deficiency was investigated using the same methods, but with 17-hydroxy-progesterone (Steraloids; 1, 2, 6, 7 [^{3}H]-17α-hydroxy-progesterone, American Radiolabeled Chemicals, St. Louis, MO) as the substrate; trilostane was not used in this study. For both studies, 10 μM substrate (1% hot, 99% cold) was combined with trilostane (when used) and evaporated to dryness under air. After evaporation, substrate (and trilostane) was resuspended in 460–467 μl assay buffer (50 mM KPO_4 and 1 mM EDTA). Microsomal protein (20 μg) was added to the resuspension, and to maintain a constant supply of reducing equivalents (dihydronicotinamide adenine dinucleotide phosphate), a generating system consisting of 17 mM glucose-6-phosphate, 2 mM β-nicotinamide adenine dinucleotide phosphate, 1 mM dihydronicotinamide adenine dinucleotide phosphate, and 1 U glucose-6-phosphate-dehydrogenase was also added. After incubation for 1 h in a 37 C water bath, 0.5 ml buffer was added. Extraction of steroids in the entire assay was performed with 10 ml methylene chloride. All samples were spun for 10 min at 2000 $\times$ *g*; the aqueous phase was then removed and the organic phase was poured into a clean tube and evaporated to dryness. All samples were resuspended in 1 ml HPLC-grade methanol and subjected to radio-HPLC. 17α-Hydroxylase activity was determined from the sum of 17α-hydroxylated metabolite (11-deoxycortisol, 17-hydroxy-pregnenolone, DHEA) radioactivity after conversion to amount of substrate metabolized.

Radio-HPLC

All solvents (including water) were of HPLC grade and all chemicals were of analytical grade. Radio-HPLC analysis of the samples was performed with an HP1100 liquid chromatographic system (Agilent Technologies, Menlo Park, CA) interfaced with a Radiomatic Model 500TR Flow Scintillation Analyzer (Packard Instrument Co., Meriden, CT). The HPLC system consisted of a solvent degasser, binary pump, autosampler, column heater, and a variable wavelength detector. Scintillation fluid (Ultima-flo, Packard Instrument Co.) flow rate was 3 ml/min. Samples (10 μl) were injected onto a 250 $\times$ 4.6 mm C18 HPLC column (BetaBasic-18, Keystone Scientific, Bellefonte, PA), then eluted with a mobile phase consisting of A (84% ammonium acetate buffer:8% MeOH:8% acetonitrile) and B (42% MeOH:42% acetonitrile:16% H_2O). B was held at 20% for 5 min, increased from 20–95% by a linear ramp (1 ml/min) from 5–20 min, held at 95% from 20–40 min, reduced to 20% from 40–45 min, then reequilibrated at 20% from 45–50 min. Positive controls ([^{3}H]pregnenolone and [^{3}H]17-hydroxy-progesterone) were injected with each assay to verify the radiodetector response and retention time. When appropriate, methanol blanks were injected between samples to monitor carryover. Retention times and area-under-the-curve of the radioactive peaks were calculated using FLO-ONE software (Packard Instrument Co.).

Statistics

All values are expressed as mean ± SEM, unless otherwise stated. Dose-dependent effects of ACTH on cortisol were analyzed by one-way ANOVA followed by *post hoc* univariate F tests. Other *in vivo* steroid levels were compared by paired *t* tests. One-way ANOVA was performed on the densitometry of the adult male marmoset adrenal sections stained for P450c17 and 3β-HSD. Student's *t* test was used to determine differences in whole adrenal microsomal substrate metabolism.

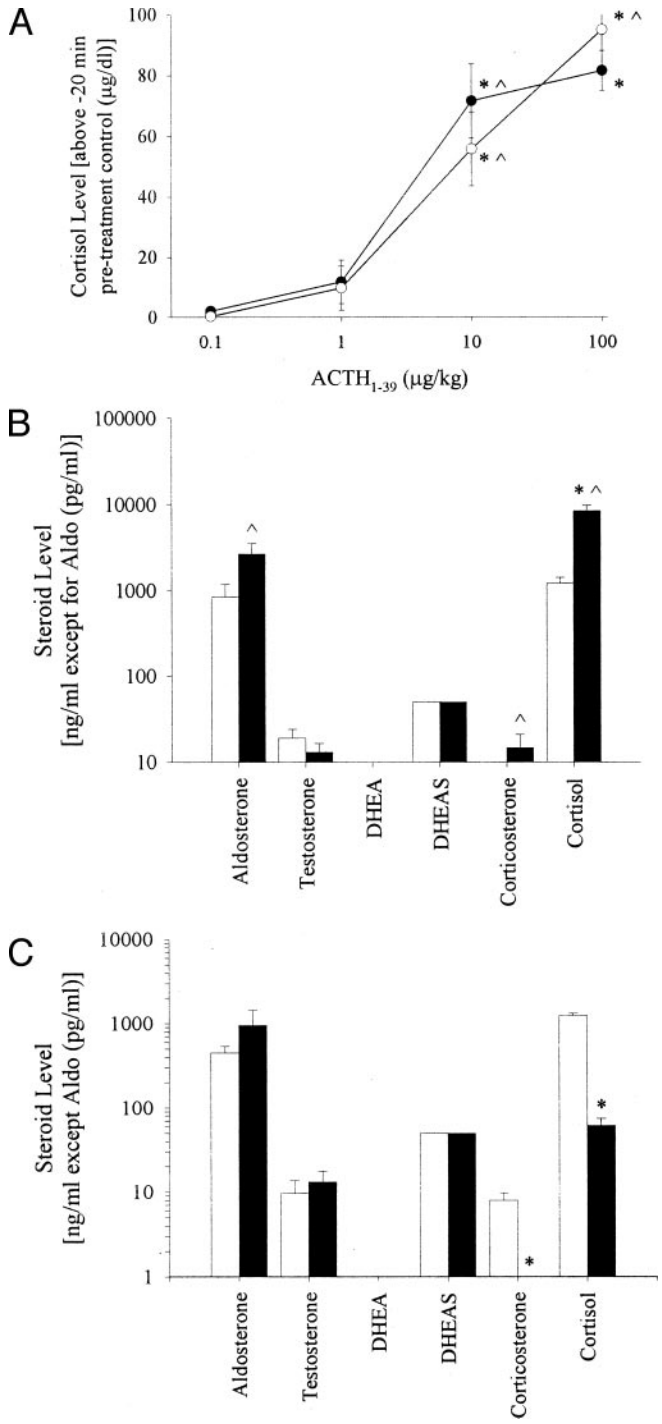

FIG. 2. A, Plasma cortisol dose-response to $ACTH_{1–39}$ at 0 (0900 h), 60 (1000 h), and 120 (1100 h) min post injection in four male marmosets, aged 38–62 months. *Black circles* represent 60-min values and open circles represent 120-min values. *, Significant difference between dose and saline control ($P < 0.05$); ^, significant difference between dose and previous dose ($P < 0.05$). B, Plasma steroid levels in response to 10 μg/kg or 100 μg/kg (DHEA only) $ACTH_{1–39}$ at 0 (0900 h) and 60 (1000 h) min post injection in the same four animals as in panel A. *Open bars*, Pre-$ACTH_{1–39}$ levels (after 5 mg/kg dexamethasome overnight treatment); *black bars*, $ACTH_{1–39}$ treatment levels. Note log scale for steroid level. *, Significant difference from pre-$ACTH_{1–39}$ level ($P < 0.05$); ^, significant difference in absolute response ($P < 0.05$). C, Plasma steroid levels in response to 5 mg/kg dexamethasone overnight treatment (basal 0900 h, dexamethasone 0900 h) in six male marmosets, aged 25–54 months. *Open bars* represent basal, and *black bars* represent dexamethasone treatment levels. Note log scale for steroid level. *, Significant difference from basal level ($P < 0.05$).

Results

Plasma steroids

Injection of $ACTH_{1–39}$ in dexamethasone-suppressed adult male marmosets resulted in a dose-dependent rise in cortisol by 60 min and 120 min, as shown in Fig. 2A. The threshold dose for $ACTH_{1–39}$ responsiveness was 1 μg/kg, and the maximal response was induced by 10 μg/kg at 60 min and by 100 μg/kg at 120 min. Pre-$ACTH_{1–39}$ DS was present only at a level below the assay sensitivity threshold of 5 μg/dl (50 ng/ml), and no elevation into the detectable range occurred in response to 10 μg/kg of $ACTH_{1–39}$ at 60 min post injection (Fig. 2B), the time of maximal cortisol response (Fig. 2A). DHEA was assayed in samples from marmosets treated with 100 μg/kg (Fig. 2B). DHEA concentrations approximated those at the sensitivity limit of the assay (0.5 ng/ml) before $ACTH_{1–39}$ treatment and did not increase significantly in response to $ACTH_{1–39}$. Plasma aldosterone and testosterone levels did not rise in response to $ACTH_{1–39}$ (1.65-fold increase and 3.2-fold decrease, respectively; Fig. 2B), confirming the specificity of the $ACTH_{1–39}$ response to the adrenals rather than the gonads. When baseline steroid levels were taken into account, however, aldosterone levels did exhibit a significant proportionate increase ($P < 0.05$), as would be expected using a supraphysiological dose of ACTH. Changes in circulating corticosterone levels mirrored those of cortisol, showing at least a 41-fold increase from below detectable levels (<0.35 ng/ml) in response to 10 μg/kg $ACTH_{1–39}$ at 60 min. These latter findings were more consistent with a ZF rather than the ZG origin of corticosterone production and secretion because they were relatively similar to the degree of cortisol elevation, but not of aldosterone. The ratio of corticosterone:cortisol was 0.0173 indicating 1.73% leakage from the cortisol pathway.

The contrasting experiment using dexamethasone-only treatment to suppress pituitary ACTH revealed a corresponding reduction in plasma cortisol levels, as expected (Fig 2C). Consistent with the results of the $ACTH_{1–39}$ challenge, DS and DHEA were again undetectable or near the limit of sensitivity, respectively. Plasma testosterone and aldosterone levels were not significantly affected by dexamethasone-only treatment, which is consistent with a gonadal origin of testosterone and with the fact that in the physiological range, ACTH is not a major regulator of aldosterone secretion. In addition, plasma corticosterone levels diminished along with cortisol, consistent with the ZF, and not the ZG, as the zone of origin for corticosterone biosynthesis.

IHC localization of steroidogenic enzymes in adult male marmosets

To compare adrenocortical enzyme expression patterns of marmosets to those of humans and rhesus, we stained fixed sections for the major steroidogenic enzymes that influence DHEA and DS synthesis. As expected, the adult

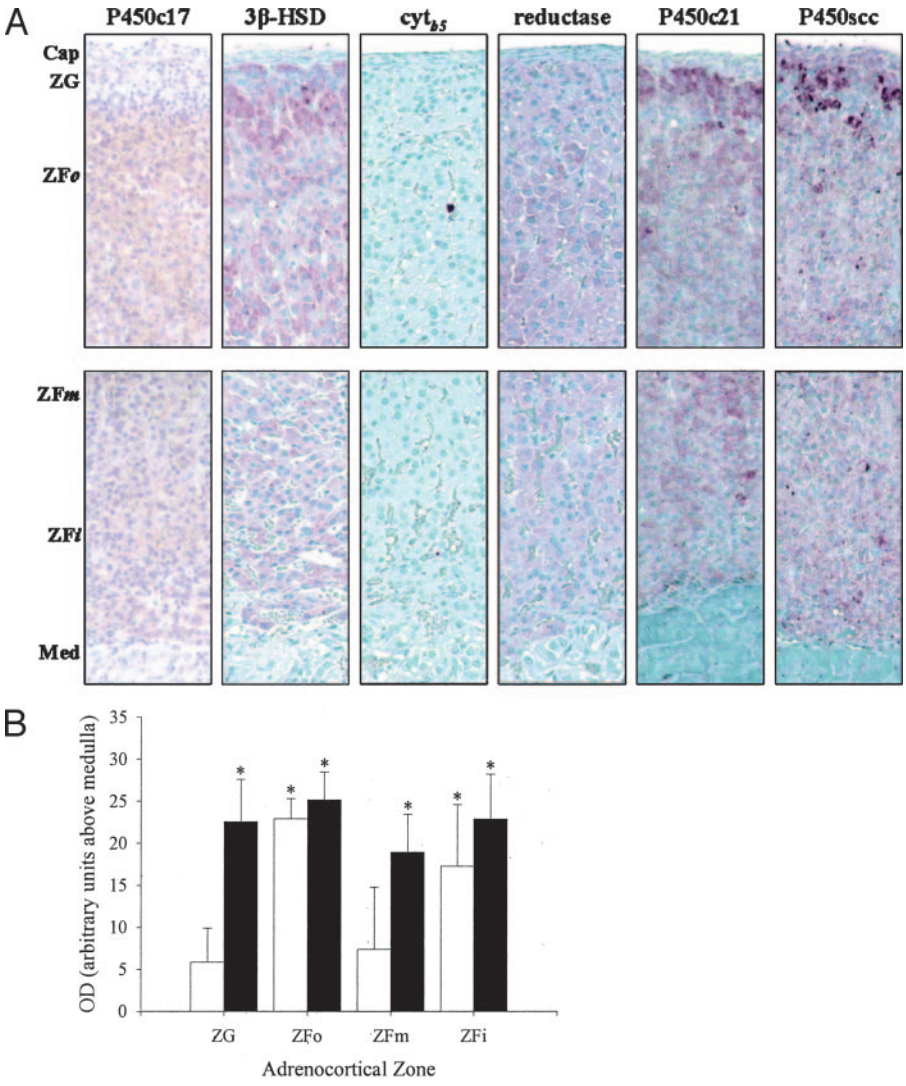

FIG. 3. A, IHC staining for steroidogenic enzymes in six adult male marmosets, aged 24–72 months. Cap (adrenal capsule) ZF*o* (outer ZF), ZF*m* (mid-ZF), ZF*i* (inner ZF). *Red* or *brown color* indicates positive stain. B, Densitometry analysis to determine relative P450c17:3β-HSD ratios. *Open bars* represent P450c17, and *black bars* represent 3β-HSD staining. *, Significant difference between enzyme staining in a specific zone and P450c17 staining in the ZG ($P < 0.05$).

male marmoset ZG was lacking in P450c17 expression, although staining was obvious throughout the rest of the adrenal cortex (Fig. 3A). Contrary to expression patterns in primates exhibiting a ZR, 3β-HSD stained throughout the adrenal cortex, including the cortico-medullary junction, and cyt$_{b5}$ staining was low or absent in the entire cortex, especially in the innermost region. Staining for cytochromes reductase, P450scc and P450c21 was observed throughout the cortex. Densitometry was performed on the sections to compare the P450c17:3β-HSD ratio (Fig. 3B). In the ZG, 3β-HSD expression was higher than P450c17 ($P < 0.05$). P450c17 was higher in the outer and inner ZF than the ZG, although expression decreased in the mid-ZF. 3β-HSD stained at similar levels throughout the entire ZF, up to the cortico-medullary junction, with no decrease in the innermost adrenocortical area.

Western analysis of adrenal microsomes

The immunoblot of marmoset adrenal microsomes confirmed the specificity of the cyt$_{b5}$ antibody, and protein expression levels of cyt$_{b5}$ were consistent with the staining trends observed by IHC (Fig. 4). Indeed, marmosets expressed P450c17 protein at similar levels to rhesus controls, but expression of cyt$_{b5}$ protein was barely detectable compared with that observed in rhesus samples.

Neonatal adrenal IHC

In view of the poor evidence for a functional ZR in adult male marmosets but previous evidence for DHEA and DS production in neonates (9), we evaluated the neonatal adrenal by IHC. As seen in the adult marmosets and other

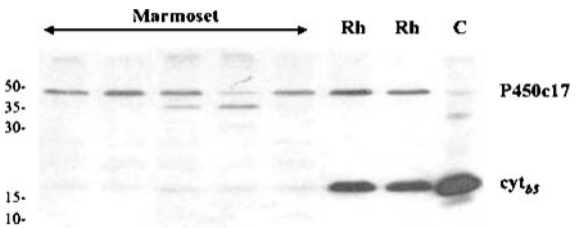

FIG. 4. Immunoblot of monkey protein probed for P450c17 and cyt$_{b5}$ from six marmoset males, aged 60–132 months, one rhesus male, aged 3 months and one rhesus female, aged 4 months. Recombinant human cyt$_{b5}$ (C), rhesus (Rh). Molecular markers are shown at *left*.

species, P450c17 was undetectable in the developing ZG in the definitive zone (DZ; Fig 5). 3β-HSD stained throughout the DZ, but expression decreased dramatically in the area expected of a FZ. The drop in 3β-HSD expression also correlated with a marked increase in cyt_{b5}, and to some extent reductase, staining in the putative FZ.

P450c17 substrate preference and relative Δ-4-lyase deficiency

No information has been published to date concerning the substrate preference of marmoset P450c17, or the presence or absence of Δ-4-lyase deficiency. Both marmoset and rhesus whole microsomal preparations showed no detectable production of progesterone, androstenedione or deoxycorticosterone, but showed substantial metabolism of pregnenolone to 17-hydroxy-pregnenolone and 11-deoxycortisol (Fig 6, A and B). The additional production of DHEA (32.49 ± 4.76% of total product) observed in male rhesus monkey preparations (Fig. 6B) was not observed in the adult male marmoset preparations (Fig. 6A). To determine more definitively whether the lack of DHEA production in adult male marmoset microsomes was a consequence of more rapid metabolism of 17-hydroxy-pregnenolone intermediate by 3β-HSD, experiments were replicated with the addition of trilostane, a 3β-HSD inhibitor. In the presence of trilostane (Fig. 6A), marmoset microsomal production of 11-deoxycortisol was reduced as expected, but this reduction was accompanied by an increase in 17-hydroxy-pregnenolone rather than an increase in DHEA.

Results determined from the net conversion of pregnenolone to 17-hydroxy-pregnenolone, 11-deoxycortisol, and DHEA showed 17α-hydroxylase activities to be similar for rhesus and marmoset preparations (160.77 ± 6.45 nmol/mg·h *vs.* 144.02 ± 5.84 nmol/mg·h, respectively; $P = 0.0673$; Fig. 6D). In the presence of trilostane, apparent 17α-hydroxylase activity was increased in both rhesus and marmoset microsomes (pregnenolone to 17-hydroxy-pregnenolone and DHEA, Fig. 6D).

A relative Δ-4-lyase deficiency can be inferred in both marmosets and rhesus from the extensive conversion of pregnenolone to 11-deoxycortisol without any apparent androstenedione production (Fig. 6, A and B) but, in view of the importance of this metabolic step in determining the control of C19 steroid production, we sought to confirm it independently. Addition of 17-hydroxy-progesterone to adrenal microsomes did not yield any androstenedione production in either marmoset or rhesus preparations (Table 1).

Discussion

We have confirmed in this study that circulating levels of DHEA and DS in adult male common marmosets are much lower than in humans (9), suggesting either very poor lyase activity relative to hydroxylase in the adrenal cortex, or the lack of a significant ZR. In contrast to such differences between marmosets and humans, marmosets share with humans many important features of adrenal steroidogenic pathways. Only certain higher mammals express P450c17 in the adrenal, and such enzyme expression is essential for cortisol biosynthesis in the ZF. Of these cortisol-secreting species, only a subset—nonhuman primates, cows, sheep, and goats—show the same deficiency in Δ-4–17,20-lyase activity observed in humans. This latter point is important in generating efficient cortisol biosynthesis without excessive C19 steroid production by the ZF (10). In addition, the relative degree of activation of cortisol release appears similar in marmosets to that in Old World primates and humans, as reflected by the use of marmosets as models for human adrenocortical function, stress, and psychopathology (3, 20–22). Marmosets are, therefore, one of a limited number of species that may be appropriate as a model for steroidogenesis in the human adrenal, and may be unique in that the effective absence of a ZR permits more straightforward interpretation of experimental manipulation of adrenal function at the endocrinological and molecular levels.

Before that can be achieved, however, it was first necessary to establish definitively whether the marmoset has a func-

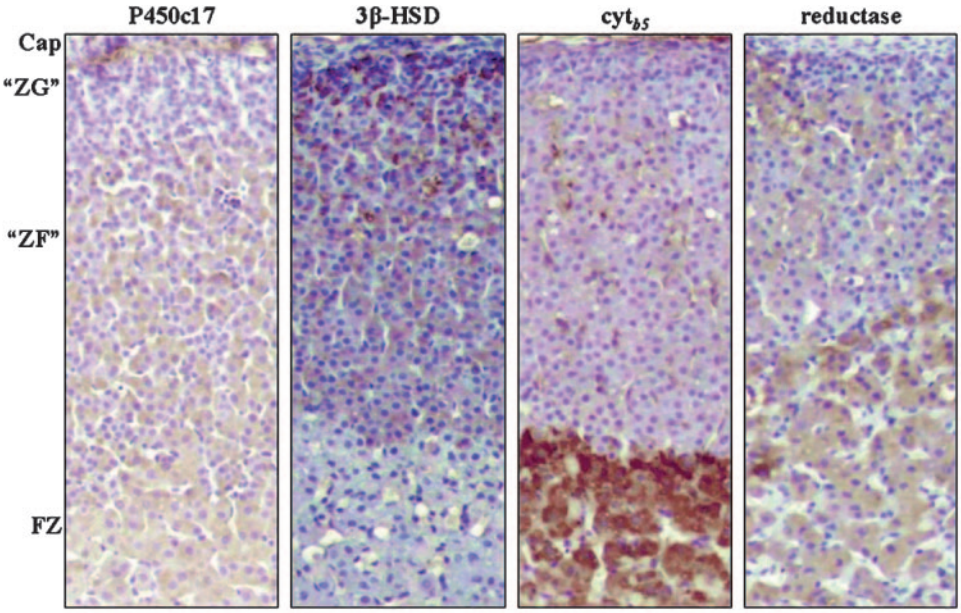

FIG. 5. IHC staining for steroidogenic enzymes in five male and one female neonatal marmosets, aged 1 d. *Red* or *brown color* indicates positive stain. Cap, Adrenal capsule; "ZG", ZG of the DZ/neocortex; "ZF", ZF of the DZ/neocortex.

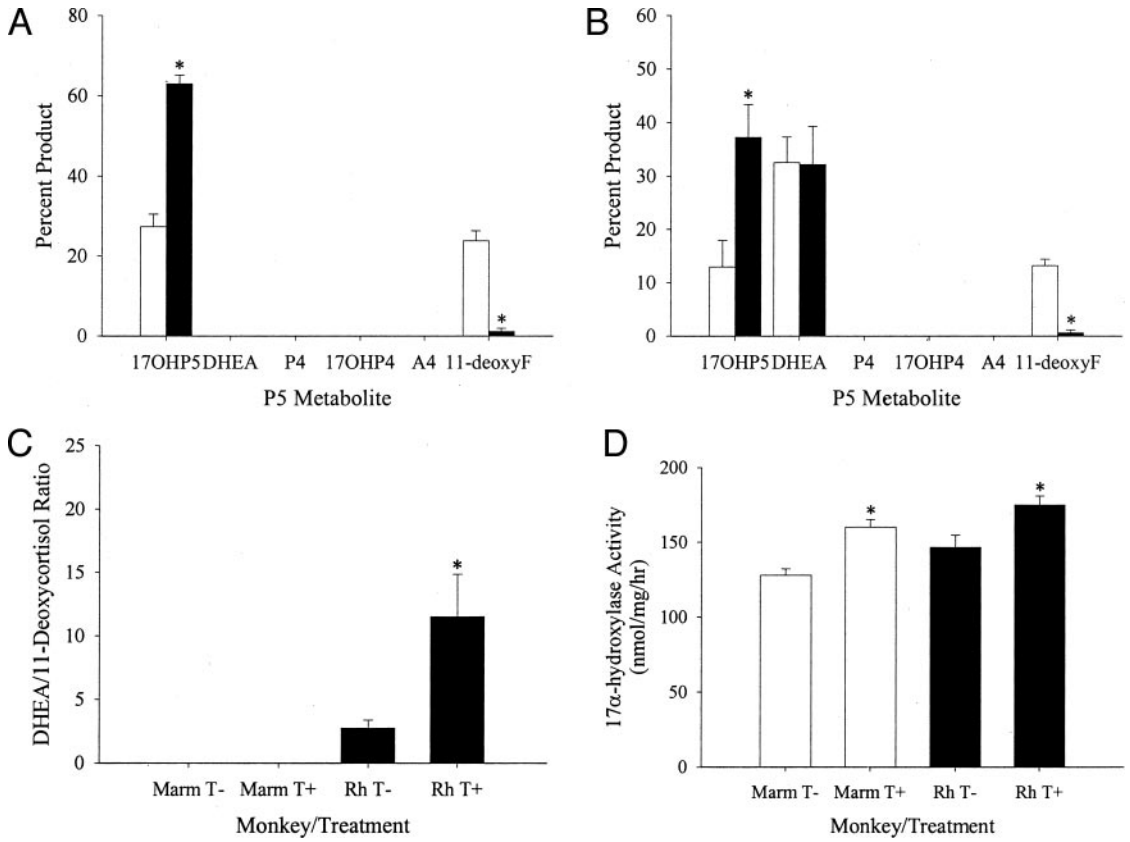

FIG. 6. Radio-HPLC detected metabolites of pregnenolone substrate addition to whole adrenal microsomal preparations (marmosets-see Fig. 4; six rhesus males, aged 3–96 months). 17OHP5 (17-hydroxy-pregnenolone), P4 (progesterone), 17OHP4 (17-hydroxy-progesterone), A4 (androstenedione), 11-deoxyF (11-deoxycortisol), T (trilostane). *Bars* are expressed as group means ± SEM (A, marmoset) and (B, rhesus). *Open bars* represent the absence and *black bars* represent the presence of trilostane. C, DHEA/11-deoxycortisol ratios calculated from A and B. Ratios are shown without (−) or with (+) trilostane. *Open bars* represent marmoset and *black bars* represent rhesus ratios. *, Significant difference upon trilostane treatment ($P < 0.05$). D, Comparative 17α-hydroxylase activities in marmoset and rhesus microsomes. Activities are shown without (−) or with (+) trilostane. *Open bars* represent marmoset and *black bars* represent rhesus activity. *, Significant difference upon trilostane treatment ($P < 0.001$).

tional ZR in the adrenal cortex because the presence of such a zone has implications for nonovarian sources of estrogen and alterations in ovarian function (10). It was also desirable to know more about the substrate preferences of 17α-hydroxylase and whether there was any associated Δ-4-lyase deficiency because these two factors are key determinants of the species-specific control of both cortisol and C19 steroid biosynthesis (10).

We performed our studies in adult male marmosets to avoid the modulating effects of the ovarian cycle and female social status on adrenocortical activity (5–7, 22). We confirmed that $ACTH_{1-39}$ increased cortisol in a dose-dependent manner in males similar to findings reported for female marmosets (22). Additionally, endogenously low levels of DHEA and DS did not respond to $ACTH_{1-39}$, which corresponded with the prior findings of Levine *et al.* (9), and dexamethasone suppression yielded no detectable change in plasma DHEA or DS levels. Our *in vivo* data appear consistent with the absence of any C19 steroid-secreting ZR that is responsive to ACTH. The many-fold higher levels of cortisol than corticosterone suggests that 17α-hydroxylase in adult male marmosets prefers pregnenolone as a substrate, and that conversion to 17-hydroxy-pregnenolone is the initial step of cortisol biosynthesis, *i.e.* as in humans, but not cows (10). The synthesis of abundant DHEA and DS requires preferential action of 17α-hydroxylase on pregnenolone (Fig 1). Thus, the lack of any measurable increase in DHEA or DS in adult marmosets is not due to a lack of substrate preference by 17α-hydroxylase for pregnenolone.

In human and rhesus adrenals, another hallmark of a functional ZR is coexpression of P450c17 with an elevated level of cyt_{b5}, and a P450c17: 3β-HSD ratio high enough to favor C19 steroid production (10). Indeed, the onset of C19 production at adrenarche has been associated with a marked reduction in 3β-HSD expression in the innermost adrenocortical area (11–13). Individual variation is seen, such that sometimes 3β-HSD is completely undetectable in the ZR, but, more commonly, a dramatically reduced level of 3β-HSD is seen in the ZR relative to the ZF. In this study, we demonstrated that marmosets, similar to humans and rhesus, do not express P450c17 in the ZG, but show P450c17 expression throughout the ZF up to the cortico-medullary junction. There was, however, no apparent or significant decrease in 3β-HSD staining in the innermost adrenocortical region, in contrast to that previously noted in humans and rhesus monkeys (11,13–14, 23). Additionally, although cyt_{b5} has been

TABLE 1. Products of whole adrenal microsomal conversion of 17OHP4 substrate as detected by radio-HPLC

	17OHP4	11-Deoxycortisol	A4
Rhesus			
1	49.08	44.69	0.00
2	16.34	70.11	0.00
3	21.82	67.92	0.00
4	30.12	53.49	0.00
5	55.18	35.70	0.00
6	52.89	32.50	0.00
7	3.24	82.27	0.00
Mean	32.67	55.24	0.00
SEM	7.62	7.11	0.00
Marmoset			
1	4.42	95.58	0.00
2	6.24	93.76	0.00
3	6.46	87.19	0.00
4	6.27	84.00	0.00
5	4.76	90.22	0.00
6	6.86	93.14	0.00
Mean	5.84	90.65	0.00
SEM	0.41	1.79	0.00
P value	$P < 0.01$	$P < 0.001$	

Values are listed as percent of total products detected. For marmosets used, see Fig. 4. For rhesus monkeys used, see Fig. 6 (with the addition of a 4-month-old male).

localized to the C19 steroid-secreting ZR in both humans (15, 24–25) and rhesus monkeys (14), and is thought to enhance 17,20-lyase activity through allosteric interactions with P450c17 (26), cyt_{b5} was largely undetectable throughout the marmoset adrenal cortex, specifically in the innermost region. Thus, our findings for 3β-HSD and cyt_{b5} in adult male marmoset adrenals are consistent with the absence of any functional ZR in these animals.

Our marmoset and rhesus monkey adrenal microsome substrate metabolism experiments provide functional evidence that adult male marmosets do not possess a functional C19 steroid-secreting ZR. Marmoset adrenal microsomes exhibited abundant 17α-hydroxylase activity, equal in magnitude to that of rhesus monkeys but, unlike the latter, showed no evidence of significant DHEA synthesis, even in the presence of trilostane. Although the use of microsomes both enriches the enzyme and simplifies interpretation of results due to removal of mitochondrial enzymes, microsomal studies are not without their limitations and it is important to consider whether the results might be artifactual. In general terms, the lack of DHEA synthesis observed in a marmoset microsomal preparation could be attributed to an inability to metabolize the given substrate, 17α-hydroxylase activity deficiency, 17,20-lyase activity deficiency, or reduced expression of P450c17 protein. Although DHEA was not a detectable product, marmoset adrenal microsomal preparations were capable of metabolizing pregnenolone to 17-hydroxy-pregnenolone and 11-deoxycortisol, as well as 17-hydroxy-progesterone to 11-dexoycortisol, suggesting reduced 17,20-lyase activity relative to rhesus.

Some validation of our microsomal methodology is given by the use of a positive control. DHEA was synthesized by the rhesus monkey adrenal microsomal preparations from pregnenolone, showing that DHEA synthesis can be achieved with the experimental design and substrate concentrations used in this study. Upon addition of trilostane in rhesus microsomes, the DHEA:11-deoxycortisol ratio increased significantly ($P < 0.05$, Fig. 6C). There was a significant increase in 17-hydroxy-pregnenolone in marmoset and rhesus preparations, confirming inhibition of conversion to Δ-4 steroid products in both species. The lack of a corresponding increase in DHEA synthesis in marmosets by this method suggests that metabolism via 17,20-lyase is already saturated (and thereby inefficient) and that inhibition of 3β-HSD alone will not guarantee a significant increase in DHEA output. This shows clearly that the lack or inhibition of 3β-HSD is not the single determining factor for efficient 17,20-lyase activity in the adrenal.

The finding that 17,20-lyase activity was detectable in marmoset adrenal microsomes by a direct radiometric assay, albeit at 10% of that in rhesus monkey adrenal microsomal preparations (data not shown), is most likely due to the increased sensitivity of the radio-metric 17,20-lyase activity assay compared with the product analysis of radio-HPLC. It is equally possible that the failure to detect DHEA resulted in part from its further metabolism to other products such as DS that would not have been recovered during the organic extraction process. Regardless, it is clear that the 17,20-lyase activity of marmoset adrenal microsomes is substantially lower than that of rhesus monkey adrenal microsomes, even though P450c17 protein expression was similar.

Adrenal Δ-4-lyase activity was examined using 17-hydroxy-progesterone as the substrate for the marmoset and rhesus monkey adrenal microsomes. In neither case did adrenal microsomal preparations metabolize 17-hydroxy-progesterone to detectable levels of androstenedione. Although it is possible to interpret this finding as support for a Δ-4-lyase deficiency, such as seen in bovine and humans (27), it is also possible that the affinity of 21-hydroxylase for 17-hydroxy-pregnenolone is much greater than that of 17,20-lyase, thereby resulting in the complete conversion of 17-hydroxy-progesterone to 11-deoxycortisol (10). It is noteworthy that in our HPLC analysis of steroid metabolites, 21-hydroxylase activity was more apparent in microsomes from marmosets than in those from rhesus monkeys (Fig. 6, A and B). Future cloning and functional expression of marmoset P450c17 cDNA, such as described for rhesus monkey P450c17 (28) will be necessary to determine conclusively whether marmosets are truly Δ-4-lyase deficient or whether it is competition with 21-hydroxylase that results in a physiologic Δ-4-lyase deficiency. Knowledge of which mechanism is responsible for the observed Δ-4-lyase deficiency is necessary before the marmoset can be used as a model for human adrenal and ovarian steroidogenesis studies.

The notable differences in ZR function of marmosets compared with humans and rhesus beg the question of whether there may also be differences in fetal adrenal development. Our examination of neonatal marmoset adrenals showed clear evidence for a FZ, again correlating with the findings of Levine *et al.* (9) using histochemical staining, and Suzuki *et al.* (15) and Parker *et al.* (29) using immunospecific and mRNA-specific methods. We conclude that marmosets resemble humans, more so than does the rhesus macaque (30), in possessing a C19 steroid-

secreting region at birth, suggesting similar intrauterine development, and presumably parturition, processes. Therefore, we further conclude that the differences observed in adrenal zonation between humans and marmosets during adulthood develop after birth.

The fact that IHC indicated regions of high cyt_{b5} expression in the innermost area of the neonate adrenal at a time when significant DS production is known to occur also makes it tempting to speculate that cyt_{b5} may be a key factor in determining, or permitting, 17,20-lyase activity in the marmoset. It could be argued, however, that the adult male marmoset adrenal also shows staining of 3β-HSD up to the corticomedullary junction, and the neonatal adrenal shows a drop in 3β-HSD in the putative FZ, otherwise rich in cyt_{b5}, suggesting that it is the presence of 3β-HSD in adult marmoset adrenals that is preventing 17,20-lyase from using its substrate. Nonetheless, microsomal activity assays performed in the presence of trilostane failed to stimulate lyase activity. Thus, the lack of apparent lyase activity in adult males does not appear to be solely a function of competing 3β-HSD. Further studies will be necessary to determine whether cyt_{b5} is indeed a direct determinant of lyase activity or whether increases in its expression are permissive or associated with an increase in lyase activity through another mechanism such as P450c17 phosphorylation (31).

In conclusion, we have shown that adult male marmosets lack significant circulating DHEA and DS because they lack a functional zona reticularis. Despite this difference from humans and rhesus monkeys, the development of the marmoset *in utero* involves the development of a FZ, capable of C19 steroid biosynthesis, that regresses before adulthood (9). Preliminary analyses of steroid responses *in vivo* and of microsome preparations *in vitro* suggest that marmoset 17α-hydroxylase prefers pregnenolone as a substrate and shows comparatively poor Δ-4-lyase activity. The existence of a primate model with similar fetal adrenal development to humans, but lacking a clearly defined or functional ZR, may prove useful for a number of studies. Examples include studies of ZF function or of ovarian steroidogenesis without the added complication of ZR-derived steroids, and examination of factors (increases in cyt_{b5} or decreases/inhibition of 3β-HSD) that may be able to induce a ZR phenotype in the ZF. Such studies may in turn illuminate as-yet poorly understood phenomena, including adrenarche and adrenopause, and poorly understood endocrinopathies that relate to control of 17α-hydroxylase activity, factors controlling androgen production and factors associated with a lack of adrenal androgen production such as mutations associated with isolated 17,20-lyase deficiency, which may then give insight into possible clinical treatment.

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