A Mechanism for Virilization of Female Spotted Hyenas in Utero

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Female spotted hyenas exhibit male-like genitalia and dominance over males. Hyena ovarian tissues incubated in vitro produced large quantities of the steroid hormone precursor androstenedione. The activity of aromatase, which converts androstenedione to estrogen, was one-twentieth as great in hyena versus human placental homogenates. In comparison, the activity of 17β-hydroxysteroid dehydrogenase, which converts androstenedione to testosterone, was equal in the two homogenates. The limited aromatase activity may allow the hyena placenta to convert high circulating concentrations of androstenedione to testosterone, which results in virilization of the fetal external genitalia and possibly destruction of fetal ovarian follicles. Androstenedione production by residual ovarian stromal cells during reproductive life accounts for the epigenetic transmission of virilization in female spotted hyenas.

Female spotted hyenas (*Crocuta crocuta*) have attracted attention since antiquity because of their highly masculinized external genitalia, a feature that promulgated the concept of hermaphroditism (*1*). The labia are fused to form a pseudoscrotum and the clitoris is enlarged to form a male-like phallus through which the urogenital sinus traverses (*2–4*). Female spotted hyenas are also heavier than male hyenas (*2, 5*), engage in as much rough-and-tumble play (6), are more aggressive than males (*7*), and are dominant in competitive situations (*8*). This array of morphological and behavioral characteristics suggests that the females are exposed to high concentrations of androgens during fetal life (*9–11*). Attention has focused on the fetal ovaries as the source of virilizing androgens because of their unusually low number of follicles and abundant amounts of stromal tissue (*2*).

On the basis of hormone measurements, earlier investigators concluded that testosterone (T) from the fetal ovaries virilizes female spotted hyenas (*12*). We have noted that the concentration of plasma androstenedione (A), an inactive precursor of both T and estrogens, is higher in nonpregnant female spotted hyenas than in adult males (*5, 13*). Furthermore, the concentration of plasma T, which is lower in adult females than in males, increases during pregnancy until it exceeds that of males, whereas the plasma A concentration remains constant or rises slightly (*14*). Together with the finding of a T concentration gradient on both the maternal and fetal sides of the placenta (*14*), these data suggested that the placenta is the source of T during spotted hyena pregnancy. To elucidate the virilizing mechanism or mechanisms, we first examined steroid production by ovarian tissues obtained from pregnant spotted hyenas (*15*).

Separated corpora luteal and stromal fragments from a pregnant spotted hyena ovary were incubated in vitro, and steroid release into the medium was monitored by radioimmunoassay (*14*). Progesterone was a major product secreted by the corpus luteum, and estrogen (at low levels) was released only by stromal tissues (Fig. 1). Large amounts of A but very small amounts of T were produced by both luteal and stromal tissues. Stimulation of steroidogenesis, by the addition of ovine luteinizing hormone (LH) to the incubation medium, increased A production by the stromal tissue. Similar results (A:T ratios of 20:1 and 300:1) were obtained when we measured androgen production by unseparated ovarian fragments from two other pregnant animals. When we incubated ^{3}H-labeled progesterone with separated ovarian fragments and analyzed the radiolabeled products by high-performance liquid chromatography, large amounts of [^{3}H]A but little [^{3}H]T was formed (*16*). However, the yield of [^{3}H]A [counts per minute (cpm) per milligram of protein per 4 hours] was 20 times greater with stromal tissue than with corpus luteal tissue, regardless of whether LH was present (luteal = 8,365 cpm; luteal plus LH = 14,723 cpm; stromal = 177,278 cpm; stromal plus LH = 267,775 cpm). Together, these results demonstrate that ovarian stromal tissue produces large amounts of A rather than T and account for the high concentration of plasma A in female spotted hyenas (*5, 13*).

Next, we measured the activity in spotted hyena and human placental homogenates of aromatase, which catalyzes the conversion of A to estrogens, and 17β-hydroxysteroid dehydrogenase (17β-HSD), which promotes conversion of A to T. In typical experiments (Fig. 2), the apparent maximum velocity (V_{max}) of aromatase in spotted hyena placental homogenates (42 pmol per milligram of protein per hour) was approximately 1/20 the value obtained for human placental homogenates (990 pmol per milligram of protein per hour), whereas the apparent Michaelis constants (K_m) were similar (0.33 and 0.91 μM, respectively). The difference in mean aromatase V_{max} values obtained with four human placentas [1604 ± 193 pmol per milligram of protein per hour (mean ± SEM)] and six spotted

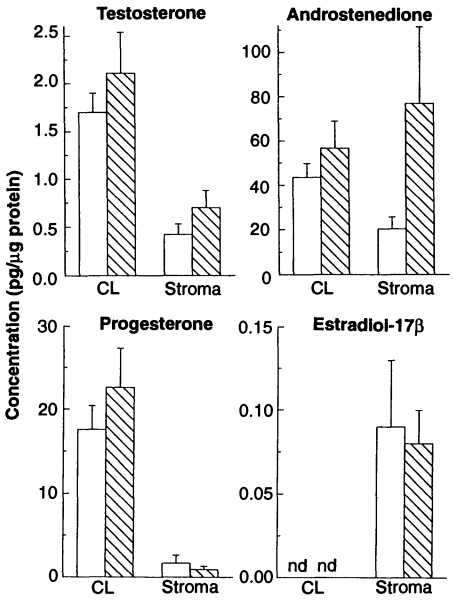

Fig. 1. Production of steroids by tissue fragments of corpus luteum (CL) and stroma from the ovary of a late-term pregnant spotted hyena. Samples of each type of tissue were dissected under a microscope, transferred to ice-cold Dulbecco's modified medium (DME; Gibco), and minced into pieces 0.5 to 1 mm in size. Minces (four replicates per treatment group) were preincubated in DME for 1 hour at 37°C and then transferred into 0.5 ml of fresh DME with or without ovine LH (100 ng/ml) (NIH-LH-S16) for an additional 2 hours at 37°C. Incubation fluids were removed and frozen at −20°C until assayed. Tissue protein content was determined with the Bradford reagent (*27*). Steroid levels in the media were measured by radioimmunoassay as described (*14*). Error bars represent the mean + 1 SEM; nd, not detected; open bars, control; hatched bars, LH.

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hyena placentas (80 ± 2.0 pmol per milligram of protein per hour) was highly significant ($P < 0.01$), but the mean apparent K_m values were not significantly different ($P > 0.1$). In contrast, the apparent V_{max} values for 17β-HSD in spotted hyena and human placental homogenates (28.6 and 18.6 nmol per milligram of protein per hour, respectively) were similar and much higher than the values for aromatase (Fig. 3). The apparent K_m values for hyena and human placental 17β-HSD were virtually identical (4.16 and 4.20 μM, respectively) and five- to tenfold higher than the values obtained for aromatase. Estimates of both V_{max} and K_m values for 17β-HSD were reproducible in experiments with six hyena and four human placentas, and no statistically significant differences were found between the species. The sex of the fetus had no effect on the activity of either aromatase or 17β-HSD in experiments with five male and six female hyena placentas. Although caution must be taken in extrapolating to the in vivo situation, these findings indicate that T is the major product formed from circulating A in the spotted hyena placenta.

These results also suggest that the low aromatase activity in the spotted hyena placenta allows conversion of the high circulating level of A to T by the apparently more abundant 17β-HSD. Thus, the ovaries and placenta act together to generate the high T concentrations in fetal plasma that in all likelihood cause virilization of the external genitalia of the female spotted hyena at the time of sexual differentiation. Moreover, high A production by the ovaries during reproductive life provides an epigenetic mechanism for transmitting virilization from one generation of females to the next in the spotted hyena and perhaps other species. However, the mechanism or mechanisms responsible for the unusually small number of follicles and large amount of stromal tissue found in spotted hyena ovaries at birth, features that we confirmed in the present study, remain unexplained.

Studies in a rodent model are especially relevant to this question because the hormonal milieu closely resembles that found in the hyena fetus. Estrogen treatment of immature, hypophysectomized rats stimulates granulosa cell growth and development of ovarian follicles to the preantral stage. However, within 72 hours of administering T to such rats, the number of normal follicles is greatly reduced as a result of pyknosis and death of granulosa cells and their dependent oocytes (*17*), a process that is known as follicular atresia. Thecal cells from the follicle wall, which normally provide A for granulosa cells to synthesize estrogen, survive and, together with interstitial cells, form the residual stromal tissue. A direct cytotoxic effect of T in such experiments is possible, as both rat (*18*) and human (*19*) ovarian granulosa cells express androgen receptors. Thus, the high plasma T levels in the spotted hyena fetus may cause massive ovarian follicular atresia as well as virilization of the external genitalia.

A recent example of virilization of both mother and female fetus during an otherwise normal human pregnancy was shown to be caused by a genetic mutation that resulted in an inactive form of placental aromatase (*20*). Consequently, dehydroepiandrosterone sulfate (DHEAS), a circulating estrogen precursor secreted by both the maternal and fetal adrenal glands in humans (*21*) and other primates, was converted to A by normal placental steroid sulfatase and 3β-HSD-5-isomerase and then to T by 17β-HSD. Virilization of the human female fetus also occurs in congenital adrenal hyperplasia (CAH), a condition in which placental aromatase is normal but adrenal DHEAS production is elevated as a result of a deficiency in cortisol biosynthetic enzymes (*22*). We postulate that high plasma concentrations of DHEAS give rise to A concentrations in the placenta that exceed its aromatizing capacity, resulting in T formation as in pregnant spotted hyenas. In milder forms of CAH, virilization does not occur until adolescence or puberty (*23*). Affected females have polycystic ovaries and high plasma A concentrations, suggesting that androgen-induced follicular atresia occurred in utero after differentiation of the genitalia was complete.

Interestingly, spotted hyena ovaries histologically resemble ovaries from women with polycystic ovarian syndrome (PCOS), a disease that is not associated with defects in adrenal steroidogenesis (*24*). Women with PCOS and the more severe condition known as hyperthecosis, in which ovarian follicles are markedly reduced in number, have abnormally high concentrations of plasma A and LH and develop hyperandrogenism around puberty (*25*). The etiology of PCOS remains unknown, although many hypotheses including some that propose a genetic basis (*26*) have been put forth. The

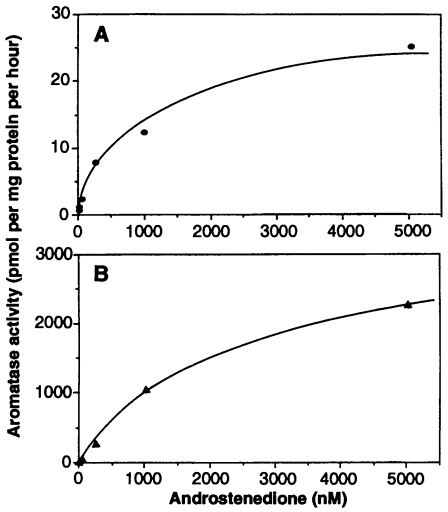

Fig. 2. Aromatase activity in (**A**) spotted hyena and (**B**) human placental homogenates. After removal of membranes and large vessels, placental tissue was minced with a razor blade and homogenized in ice-cold phosphate-buffered saline (1/10 w/v). Aromatase activity was measured in whole homogenates by a radiometric procedure in which [19-^{3}H]A was used as substrate (*28*). Protein in placental homogenates was measured by the Bradford method (*27*). Double reciprocal plots of the data were prepared and regression lines computed by the sum of least squares method. Species difference in aromatase activities was analyzed by the Mann-Whitney U test (*29*). (A) V_{max}, 42 pmol per milligram of protein per hour; K_m, 0.2 μM. (B) V_{max}, 990 pmol per milligram of protein per hour; K_m, 0.9 μM.

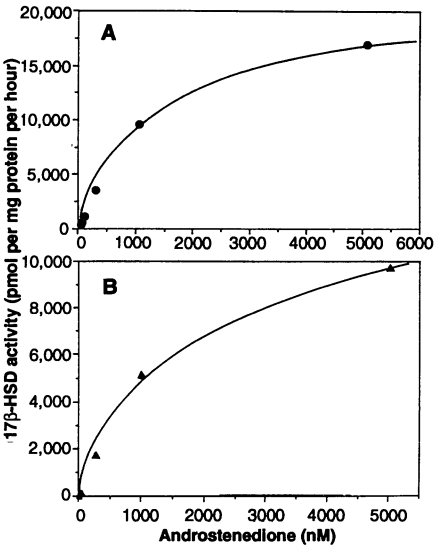

Fig. 3. Activity of 17β-HSD in (**A**) spotted hyena and (**B**) human placental homogenates. Metabolism of A to T and other metabolites was examined as described in Fig. 2 and (*30*). Briefly, 80 μl (0.3 to 0.4 mg of protein) of placental homogenate was diluted to 1 ml in order for the reaction rate to remain linear for more than 10 min. At the completion of 10-min incubations with [19-^{3}H]A and various amounts of nonradioactive A, the tubes were frozen, stored, and later extracted twice with 1 ml of ethyl acetate. To an aliquot of the pooled organic phase, which contained ~80,000 cpm, was added 80 μg each of nonradioactive T, A, dihydrotestosterone, 5α-androstenedione, and 5α-androstane-3β,17β, diol. The yield of radioactive T was measured after separation of steroids by thin-layer chromatography and elution with methanol. (A): V_{max}, 28.6 nmol per milligram of protein per hour; K_m, 4.16 μM. (B): V_{max}, 18.9 nmol per milligram of protein per hour; K_m, 4.5 μM.

common ovarian abnormalities and high plasma A concentrations suggest that PCOS may be the consequence of placental T synthesis and androgen effects on the hypothalamic-pituitary-ovarian axis late in fetal life that result in excessive LH and A secretion following puberty.

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15. The spotted hyenas were collected as infants in southwest Kenya and reared in social groups maintained at the Field Station for Behavioral Research adjacent to the University of California, Berkeley campus. Hyena ovaries and placentas were obtained from pregnancies terminated within the last 30 days of the 110-day gestational period according to University of California institutional guidelines. Details of these operations and results of experiments on plasma steroid hormone levels are described elsewhere (*14*). Human placentas were obtained from cesarean sections at term of uncomplicated pregnancies. Placentas were removed from the uterus, dissected free from fetal membranes, and washed in ice-cold phosphate-buffered saline (pH 7.4). All samples were transferred to the laboratory on ice within 2 hours of surgery.
16. Minced fragments of corpora lutea and stromal tissue were incubated with 1 × 10^6 cpm of [^{3}H]progesterone (New England Nuclear, Boston; 30 mCi/mmol) for 4 hours in 0.5 ml of DME as described in Fig. 1. Radiolabeled products were extracted twice with 1 ml of ethyl acetate. The extracts were combined, evaporated, and analyzed on a Waters high-performance liquid chromatography unit fitted with a reversed-phase column and gradient elution device. We quantified radiolabeled products by counting fractions associated with nonradioactive steroid standards. In three separate incubations, [^{3}H]A accounted for more than 85% of the products formed by stromal tissue.
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The Role of T_H1 and T_H2 Cells in a Rodent Malaria Infection

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CD4$^+$ T cells play a major role in protective immunity against the blood stage of malaria, but the mechanism of protection is unclear. By adoptive transfer of cloned T cell lines, direct evidence is provided that both T_H1 and T_H2 subsets of CD4$^+$ T cells can protect mice against *Plasmodium chabaudi chabaudi* infection. T_H1 cells protect by a nitric oxide–dependent mechanism, whereas T_H2 cells protect by the enhancement and accelerated production of specific immunoglobulin G1 antibody.

Malaria, principally *Plasmodium falciparum* malaria, affects 250 to 400 million people and causes more than 1 million deaths annually (*1*). Vaccine development would be facilitated by a better understanding of the host immune mechanisms (*2*).

P. c. chabaudi AS strain infection in the laboratory mouse is a recognized model for *P. falciparum* infection of humans. NIH mice infected with *P. c. chabaudi* (1 × 10^5 parasitized red blood cells, pRBC) develop an acute primary parasitemia that peaks on day 10 and lasts 14 to 18 days, usually followed by one more patent parasitemias (recrudescence) between days 25 and 35 after infection. Further infections are resolved more rapidly. CD4$^+$ T cells are involved in immunity to the asexual erythrocytic stages of *P. c. chabaudi*, and both T_H1 and T_H2 subsets of CD4$^+$ T cells may mediate protection (*3*).

We investigated the effect of injecting L-N^γ-monomethylarginine (L-NMMA), a specific inhibitor of nitric oxide (NO) synthase, on the course of infection (*4*). Normal mice produced significant levels of nitrate in the serum (630 ± 85 nmol/ml), the peak of which coincided with the peak of the primary parasitemia (*5*) and declined rapidly thereafter. Mice receiving L-NMMA developed a markedly increased primary parasitemia [peak of 4.8 log(pRBC/10^5 RBC)] lasting 22 days, and sera from these mice contained little or no detectable nitrate.

A panel of eight CD4$^+$ T cell clones specific for trophozoite-schizont antigens and restricted by major histocompatibility complex class II molecules was derived from mice infected with *P. c. chabaudi* (*6*). Four were T_H1 clones, secreting interleukin-2 (IL-2) and interferon-gamma (IFN-γ); the other four were T_H2 clones in that they secreted IL-4 and provided help for antibody production. Two representative clones, WEP 999 (T_H1, from day 16 of infection) and WEP 988 (T_H2, derived from a reinfected donor), were selected for functional study; both were CD3$^+$, CD4$^+$, CD8$^-$, T cell receptor (TCR) αβ$^+$ and TCRγδ$^-$.

Female adult inbred NIH mice (*7*) were thymectomized and injected intraperitoneally with rat monoclonal antibody (mAb) specific for mouse CD4 or CD8 or with normal rat immunoglobulin G (IgG) (*8*) and infected with *P. c. chabaudi*. This protocol depleted ~98% of CD4$^+$ or CD8$^+$ T cells for more than 60 days (*9*). Mice depleted of CD4$^+$ T cells suffered 75 to 90% mortality within 20 days of infection, and those that survived maintained a constant and high level of parasitemia [3.4 to 3.8 log(pRBC/10^5 RBC)] for more than 60 days. In contrast, mice depleted of CD8$^+$ T cells had a parasitemia profile indistinguishable from that of mice treated with normal rat IgG, and all mice recovered from infec-

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