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ORIGINAL PAPER

The impact of unabated stimulation by human chorionic gonadotropin on the steroid hormone environment of pregnant rats and the spontaneous expression of ovarian cysts in female progeny

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Abstract Unabated stimulation by low doses of human chorionic gonadotropin (hCG) induces ovarian cysts in pregnant rats. In order to determine the impact of these in vivo treatments on the hormonal milieu of pregnancy, and the potential impact of an aberrant cystic-ovary state during pregnancy on the resulting female offspring, pregnant rats were treated with either 0 (control), 1, or 3 IU hCG twice daily for at least 9 days, beginning on day 13 of pregnancy. Serum was harvested from control and hCG treated animals on days 15, 17, 19, and 22 of pregnancy. Control pregnant rats and animals treated with 1 IU hCG shared similar serum profiles for progesterone (P4), androstenedione (A4), 5α-androstane-3α,17β-diol (3α-diol), androsterone (A5), and estrone (E1) between days 15 and 22 of pregnancy. Testosterone serum concentrations were similar for control and 1 IU hGG-treated pregnant rats between days 15 and 19 of pregnancy; whereas, on day 22, 1 IU hGG-treated pregnant rats displayed *lower* serum testosterone than control pregnant rats ($P \leq 0.05$). In contrast, serum estradiol (E2) concentrations for 1 IU hCG-treated pregnant rats were *greater* than E2 values observed for control rats on days 15–19 of pregnancy ($P \leq 0.05$). Serum testosterone and 3α-diol values for 3 IU hCG-treated pregnant rats differed from those of control pregnant rats only on day 19 when these values were transiently greater for these hCG-treated animals compared with serum values for control pregnant rats ($P \leq 0.05$). Serum A4 values for 3 IU hCG-treated pregnant rats were elevated compared to values for control pregnant rats only on days 15 and 17 ($P \leq 0.05$). In contrast, serum E1, A5, and E2 were elevated on days 19–22, 17–22, and 15–22, respectively, in 3 IU hCG-treated pregnant rats compared to control pregnant rats ($P \leq 0.05$). *No* pups from control pregnant rats displayed ovarian cysts during the time they were observed postnatally. In contrast, 6 of 25 pups from 3 IU hCG-treated pregnant rats displayed cystic ovaries, without corpora lutea, on day 55 of age. Serum steroid concentrations for these cyst-bearing progeny were similar to those of female progeny from control pregnant rats, whereas female progeny without ovarian cysts from 3 IU hCG-treated pregnant rats displayed differences in serum steroid values from those of progeny from control pregnant rats ($P \leq 0.05$). The data support the concept that an aberrant, yet physiologic hormonal environment associated with the induction of ovarian cysts during pregnancy in rats, can lead to the spontaneous establishment of an ovarian cystic state in at least a subset of the female progeny. Further, the date suggest that tonically increased ovarian estrogen production during pregnancy, reflected by tonically elevated peripheral serum estrogen concentrations, may play a pivotal role in the etiology of an ovarian cystic state in this subset of daughters from hCG-induced, cyst-bearing pregnant rats.

Keywords Ovarian cysts · In utero exposure · Reproductive development · Pregnant rat · Pregnancy · Serum steroids · Androgens · Estrogens · Estradiol · Progeny · Daughters

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Introduction

Ovarian cysts occur spontaneously in many mammalian species and the established cystic ovary state has been studied extensively in mammals [1–11]. Yet, little is known about the actual mechanisms involved in the spontaneous development of this condition, which is the leading cause of infertility in women [1–3]. Although the mechanisms involved in the onset of PCOS remain unclear, it appears to be a multi-genetic syndrome that displays an underlying pattern of dominant inheritance.

Aging laboratory rats spontaneously develop ovarian cysts as the hypothalamic-pituitary axis becomes refractory to the long-day environment in which such animals are typically housed [6, 7]. Although it is not yet known if aging rats in the wild become refractory to their natural environment and also enter into a "constant estrus" state as they age it is known that exogenous hormonal stimuli, such as pharmacologic amounts of androgens or estrogens [9, 12–14], in addition to prolonged exposure to "long-day" light patterns [6, 7], can trigger this aberrant neuroendocrine state at the level of central mechanisms in rodents during their normal reproductive life time.

The ability of low doses of human chorionic gonadotropin (hCG) to induce large ovarian follicular cysts in pregnant rats [15, 16], progesterone-synchronized immature rats [17], and in combination with FSH in hypophysectomized immature rats [18] indicates that tonic, unabated stimulation by subovulatory doses of LH-like activity is an important factor in the development of *large* ovarian cysts in this species. Conversely, the ability to induce *small* ovarian follicular cysts in hypophysectomized (HYPOXD) rats exposed to prolonged treatment with FSH plus E2 in the presence or absence of exogenous dihydrotestosterone (DHT) [19], provides direct evidence that tonic stimulation by estrogen(s) plays a fundamental role, at least at the level of the ovary, in the induction of ovarian follicular cysts in rats.

Together, the foregoing observations led us to ask a number of interesting questions regarding the potential for a maternal ovarian cystic state [15, 16] to impact the etiology of ovarian cyst development in female progeny: (1) what is the effect of inducing ovarian cysts on the hormonal environment of pregnancy in the rat; (2) what is the effect of this maternal environment on the outcome of such pregnancies; and (3) does this maternal hormonal environment affects the expression of ovarian cysts in resulting female progeny?

When we first presented our preliminary histological findings on the potential role of the hormonal environment of pregnancy in the rat on the expression of cystic ovaries in female progeny (Annual Meeting of the Society for the Study of Reproduction, 1995, Davis, CA), little research had been performed regarding the maternal hormonal environment of pregnancy and the induction of an ovarian cystic state in female progeny. However, the effects of abnormal environments in utero on reproductive development had been documented. For example, it was known that: (1) environmental stresses during critical stages of pregnancy adversely affect the development of the female reproductive system [20, 21]; (2) androgens are required at a critical time during fetal development for normal masculinization of the hypothalamic axis [22–25]; and (3) pseudohermaphroditism is expressed in rodent progeny and some human progeny exposed to excess androgens at a critical stage during pregnancy [26, 27]. Recent studies using the long-acting pharmaceutical agent, testosterone propionate, to induce a hyperandrogenic state in pregnant sheep [10] and monkeys [11] support the concept that exposure to an inappropriate hormonal environment by the administration of exogenous steroids during pregnancy [20–27] can play an important role in determining the reproductive competence of female progeny in several mammalian species.

The data presented here extend our previous work regarding the induction of ovarian cysts in the pregnant rat [15, 16] and provide new insight regarding the impact of a maternal "cystic-ovary" state induced by subovulatory doses of hCG on: (1) the maternal peripheral steroid hormonal milieu; (2) the outcome of the affected pregnancies; (3) the spontaneous expression of ovarian cysts in female progeny exposed to this environment in utero; and (4) the peripheral steroid hormone environment observed in the daughters that spontaneously developed ovarian cysts.

Results

Impact of in vivo treatments on maternal ovarian morphology

In this series of experiments, as in our previous work with pregnant rats, the term "ovarian follicular cysts" refers to translucent follicles generally greater than 1.5 mm in diameter with well-developed thecal shells and containing just a remnant of granulosa cells. The gross ovarian morphology/histology for the 0 IU hCG-, 1 IU hCG- and 3 IU hCG-treated pregnant rats was virtually identical to the histological photomicrographs previously presented by this group for ovaries of pregnant rats undergoing this hormonal treatment regimen in vivo [15, histology not shown here]. Briefly, by day 22 of pregnancy control pregnant rats, as expected [15, 16, 33], displayed early preovulatory follicles approximately 0.75 mm in diameter; pregnant rats treated with 1 IU hCG consistently displayed precystic follicles approximately 1 mm in diameter with highly

developed thecal shells and 3–5 layers of granulosa cells; whereas pregnant rats treated with 3 IU hCG consistently displayed ovarian cysts up to 5 mm in diameter with well-developed thecal shells and only a remnant layer of granulosa cells.

Impact of unabated stimulation by hCG in vivo on the maternal hormonal environment

Figure 1 illustrates the impact of unabated stimulation by subovulatory doses of hCG on maternal serum progesterone. Mean serum concentrations of P4 for control pregnant rats ranged between 82.4 and 120.5 ng/ml on days 15–17 of pregnancy and decreased to 36.5 ng/ml by day 22 ($P < 0.05$). Mean serum concentrations of P4 for hCG-treated pregnant rats were not significantly different from those of control animals on any day tested. However, the mean serum progesterone value for animals treated with 3 IU hCG, that failed to deliver their pups by day 26 of pregnancy, was 115.6 ng/ml, which was significantly greater than that for control animals on the last day of pregnancy ($P \leq 0.05$) and similar to serum P4 concentrations observed on days 15–19 of pregnancy for all treatment groups.

Figure 2 demonstrates the impact of unabated stimulation by increasing, subovulatory doses of hCG on androgen and estrogen concentrations in maternal serum. Mean serum concentrations of A4 for pregnant rats treated with 1 IU hCG ranged between 324 and 483 pg/ml on days 15–22 of pregnancy, and were not statistically different from mean serum concentrations of A4 for control pregnant rats, which ranged between 572 and 887 pg/ml on these days ($P > 0.05$). In contrast, serum A4 concentrations for pregnant rats treated with 3 IU hCG not only were greater than those observed for control animals on days 15 and 17 of pregnancy ($P \leq 0.05$), but also were greater than the values obtained for rats treated with 1 IU hCG on days 15–22 of pregnancy ($P \leq 0.05$). Serum A4 values for pregnant rats treated with 3 IU hCG were maximal between days 15 and 17 of pregnancy (1,631 pg/ml) and decreased to 810 pg/ml by day 22 of pregnancy ($P \leq 0.05$). Further, the mean serum A4 value for animals treated with 3 IU hCG that failed to deliver their pups by day 26 of pregnancy was 503 pg/ml, which was similar to the mean serum concentration observed for these animals on day 22 of pregnancy ($P > 0.05$).

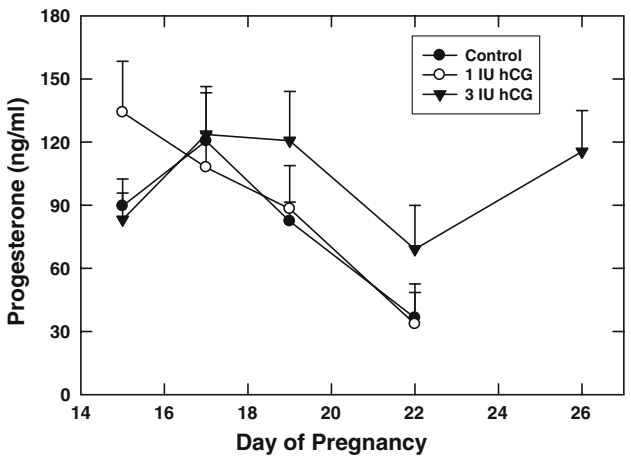

Fig. 1 Serum progesterone profiles for control pregnant rats and pregnant rats treated with increasing, subovulatory doses of hCG. Animals were treated and serum was processed and analyzed as described in "Material and methods." Data points represent the mean ± SEM for samples from 6 to 13 rats for the in vivo treatment groups on each day indicated

Mean serum testosterone concentrations for control pregnant rats ranged between 195 and 295 pg/ml on days 15–22. Mean serum testosterone values for pregnant rats treated with 1 IU hCG ranged between 112 and 163 pg/ml on these days, but were statistically less than control values only on day 22 of pregnancy ($P \leq 0.05$). Mean serum testosterone values for pregnant rats treated with 3 IU hCG were maintained between 358 and 473 pg/ml on days 15–22 of pregnancy, and differed from control values only on day 19 of pregnancy ($P \leq 0.05$). In contrast, mean serum testosterone values for pregnant rats treated with 3 IU hCG were greater than serum testosterone values for pregnant rats treated with 1 IU hCG on all days tested ($P \leq 0.05$). Further, serum testosterone was maintained at 498 pg/ml on day 26 for those animals treated with 3 IU hCG that failed to deliver their pups.

Mean serum estrone concentrations for control rats increased from 11 to 98 pg/ml between days 15 and 22 of pregnancy ($P \leq 0.05$) and were similar ($P > 0.05$) to the mean serum E1 values obtained for pregnant rats treated with 1 IU hCG, which increased from 33 to 140 pg/ml ($P \leq 0.05$) on these days. In contrast, mean serum E1 values for pregnant rats treated with 3 IU hCG were maintained at 50 pg/ml on days 15 and 17, increased to 319 pg/ml on day 19 ($P \leq 0.05$), and remained at this elevated level up to day 22 of pregnancy (465 pg/ml). By day 26, however, serum E1 decreased to 151 pg/ml ($P \leq 0.05$) for the animals that failed to deliver their pups.

Mean serum estradiol concentrations for control pregnant rats ranged between 9 and 24 pg/ml on days 15–19 of pregnancy. By day 22, these animals displayed a small, but significant increase in serum E2 to 55 pg/ml ($P \leq 0.05$). Serum E2 values for pregnant rats treated with 1 IU hCG were greater than the values observed for control rats on days 15–19 of pregnancy ($P \leq 0.05$) and ranged between 57 and 157 pg/ml on days 15–22. Serum E2 values for rats treated with 3 IU hCG were greater than those for control pregnant rats on all days tested ($P \leq 0.05$), and were similar to those for pregnant rats treated with 1 IU hCG,

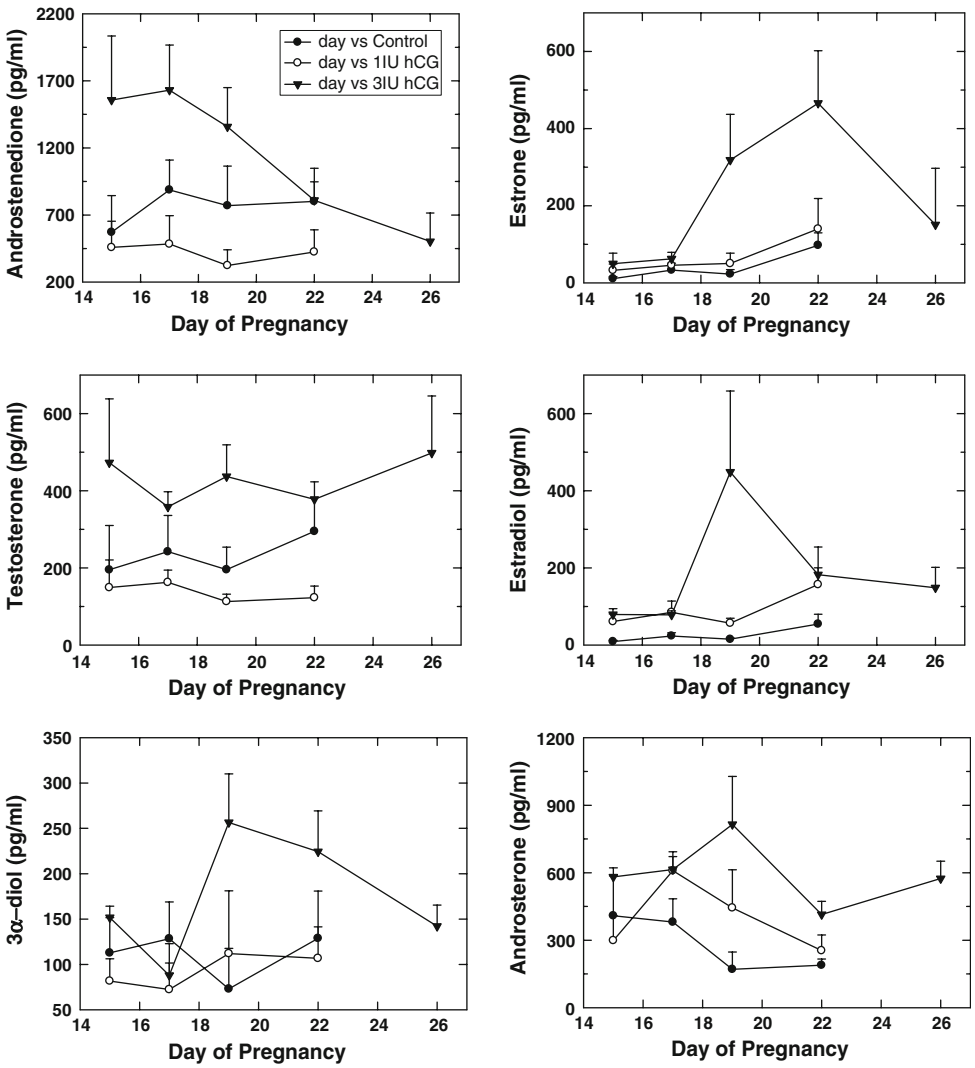

Fig. 2 Serum androgen and estrogen profiles for control pregnant rats and pregnant rats treated with increasing, subovulatory doses of hCG. Values were obtained from the serum samples used to obtain the data in Fig. 1. Data points represent the mean ± SEM for samples from 6 to 13 rats for the in vivo treatment groups on each day indicated

except on day 19 when a transient increase to 449 pg/ml was observed ($P \leq 0.05$). Further, the mean serum E2 value on day 26 (149 pg/ml) for those animals that failed to deliver their pups was similar to the value observed on day 22 for this in vivo treatment group.

Mean serum 3αdiol concentrations for control pregnant rats and pregnant rats treated with 1 IU hCG were similar on all days tested ($P > 0.05$), ranging between 73 and 129 pg/ml and between 72 and 112 pg/ml, respectively. In contrast, mean serum 3αdiol values for pregnant rats treated with 3 IU hCG increased from 88 pg/ml on day 17 to 257 pg/ml on day 19 of pregnancy ($P \leq 0.05$) and were still at this level on day 22 (224 pg/ml). Mean 3αdiol serum values for pregnant rats treated with 3 IU hCG differed from those of control pregnant rats only on day 19 ($P \leq 0.05$), and differed from those of pregnant rats treated with 1 IU hCG on days 15 and 22 ($P \leq 0.05$). The mean 3αdiol serum value on day 26 of pregnancy for animals in this treatment group that failed to deliver their pups was 142 pg/ml, and was similar to the serum value for this group on day 22 ($P > 0.05$).

Mean serum A5 values for control pregnant rats were similar to those for pregnant rats treated with 1 IU hCG throughout pregnancy and decreased for both of these treatment groups between days 15 and 22 from 409 to 171 pg/ml ($P \leq 0.05$) and from 609 to 254 pg/ml, respectively. In contrast, mean serum A5 values for rats treated with 3 IU hCG were greater than those of control animals on days 17–22 ($P \leq 0.05$) and were maintained between 415 and 815 pg/ml. Further, the mean serum A5 value for 3 IU hCG-treated pregnant rats that failed to deliver their pups was still at this level (574 pg/ml) on day 26.

Impact of maternal environment on pregnancy outcome and ovarian morphology of female progeny

Parturition generally occurs for rats on days 22–23 of pregnancy. Therefore, pregnant rats were checked twice

daily for pups starting on day 22 of pregnancy. All of the control pregnant rats throughout this series of experiments delivered their pups by the evening of day 23. In our first series of experiments, twice daily hCG treatments were given through the evening of day 25 of pregnancy. Under these conditions, none of the 3 IU hCG-treated animals and only 3 of the 1 IU hCG-treated animals underwent parturition by day 26. All pups still in utero on day 26 were dead. Since one of the objectives of this work was to determine the impact of an established ovarian cystic state on the spontaneous expression of ovarian cysts in the resulting progeny, we focused on twice daily treatments of 3 IU hCG for only nine days as performed in our earlier work with this model [15]. These animals display fully cystic ovarian follicles by the morning of day 7 of treatment, whereas pregnant rats treated with 1 IU hCG display only precystic follicles by the morning of day 9 of treatment. Approximately 30% of the animals treated with 3 IU hCG for 9 days still failed to undergo parturition by day 26 under this hormonal treatment regimen. It is interesting to note that these animals displayed cystic ovaries on day 26 even though the in vivo hCG treatments ended on day 21 of pregnancy. Viable litters were approximately 50% female for all of the in vivo treatment groups. As in our previous work, complete resorption of pregnancy was most common in control pregnant rats [15].

There was no gross morphological/histological difference between testicular cross-sections for male progeny from control pregnant rats and hCG-treated pregnant rats on any day sampled postpartum (data not shown). In contrast, 6 of 25 female progeny from pregnant rats treated with 3 IU hCG displayed large cystic and precystic follicles on day 55 of age. Figure 3 illustrates representative ovarian histological cross sections for 55-day-old noncystic female progeny from control pregnant rats and cystic female progeny from 3 IU hCG-treated pregnant rats. Fully cystic follicles in these animals possessed a mean diameter of 1.12 ± 0.10 mm. In marked contrast to daughters of control pregnant rats and *noncystic* daughters of pregnant rats treated with 3 IU hCG (not shown), ovarian cyst bearing daughters of pregnant rats treated with 3 IU hCG did not display corpora lutea, and their ovarian stromal interstitial tissue had a stimulated appearance.

Impact of exposure to maternal hormonal environment on serum steroids in female progeny

Figure 4 illustrates the mean serum P4 concentrations for daughters of control pregnant rats and daughters of pregnant rats treated with 3 IU hCG between 21 and 72 days of age. Due to the limited numbers of daughters from pregnant rats treated with 3 IU hCG, serum samples for this group were harvested beginning on day 40 of age rather than day 21. Daughters of control pregnant rats and *noncystic* daughters from pregnant rats treated with 3 IU hCG displayed similar numbers of corpora lutea. For example, at 55 days of age the ovaries of these groups displayed 14.4 + 1.0 (n = 17) and 15.4 + 0.8 (n = 19) corpora lutea, respectively.

Female progeny from control pregnant rats (control daughters) displayed mean serum P4 values of 1.5, 4.9, and 10.5 ng/ml at 21, 40, and 55 days of age, respectively. These serum P4 values were greater than 10 ng/ml for 2 of

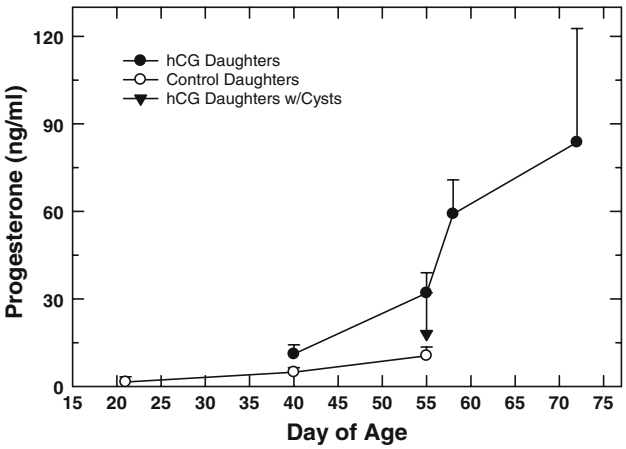

Fig. 4 Serum progesterone profiles for female progeny from control pregnant rats and 3 IU hCG-treated pregnant rats. Serum samples were obtained, processed and analyzed as described in "Material and methods." Data points represent the mean ± SEM

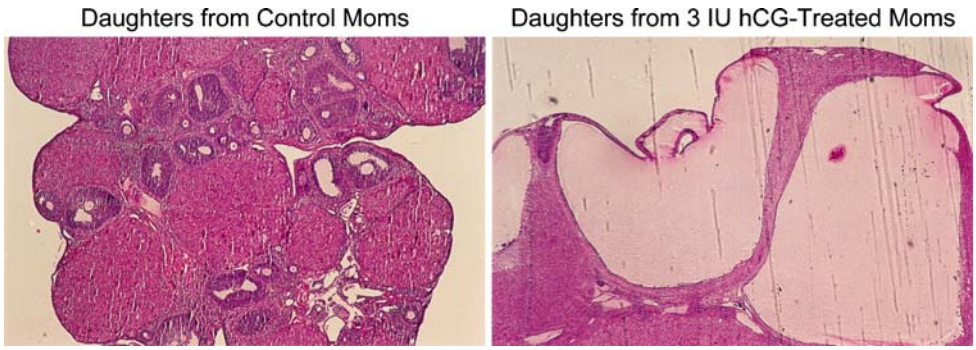

Fig. 3 Histology of ovaries from 55-day-old daughters from control pregnant rats and 55-day-old daughters bearing ovarian cysts from pregnant rats treated 3 IU hCG. Both photomicrographs were obtained using a 2.5× Zeiss Planochromat objective

17 and 6 of 17 control daughters on days 40 and 55 of age, respectively. Noncystic daughters from hCG-treated pregnant rats displayed mean serum P4 values of 11.1, 32.0, 59.1, and 83.7 ng/ml on days 40, 55, 58, and 72 of age, respectively. In contrast to control daughters, serum P4 values for *non*cystic daughters from hCG-treated pregnant rats were greater than 10 ng/ml for 7 of 15 and for 16 of 19 animals on days 40 and 55 of age, respectively. Thus, serum P4 values were greater for non-cystic daughters from hCG-treated pregnant rats than for age-matched daughters from control pregnant rats ($P \leq 0.05$) on these days of age. The six ovarian cyst bearing female progeny displayed a mean serum P4 value of 18.0 ± 14.1 ng/ml on day 55 of age. Only daughter that displayed precystic rather than fully cystic ovarian follicles possessed a serum P4 value greater than 10 ng/ml (specifically, 82.1 ng/ml) in the absence of corpora lutea. The remaining 5 animals in this group, with fully cystic ovarian follicles and no corpora lutea, displayed a mean serum P4 value of 5.16 ng/ml (not illustrated), which was similar to serum P4 values for control daughters between 40 and 55 days of age ($P > 0.05$) and markedly less than serum P4 values displayed by *noncystic* daughters from hCG-treated pregnant rats bearing corpora lutea at 55 days of age ($P \leq 0.05$).

Figure 5 depicts circulating serum androgen and estrogen concentrations for daughters from control- and hCG-treated pregnant rats. Mean serum A4 values for control daughters decreased from 245 to 51 pg/ml between day 21 and 55 of age ($P \leq 0.05$). In contrast, mean serum A4 concentrations for *noncystic* daughters from hCG-treated pregnant rats increased from 30 to 147 pg/ml between days 40 and 55 of age ($P \leq 0.05$) and remained at this level at 72 days of age (177 pg/ml). In contrast, the mean serum A4 value for the 6 daughters from the hCG-treated group bearing ovarian cysts or precysts at 55 days of age was 57 pg/ml, which was similar to values observed for control daughters on that day ($P > 0.05$) and less than values observed for their *noncystic* siblings from pregnant rats that were treated with 3 IU hCG ($P \leq 0.05$).

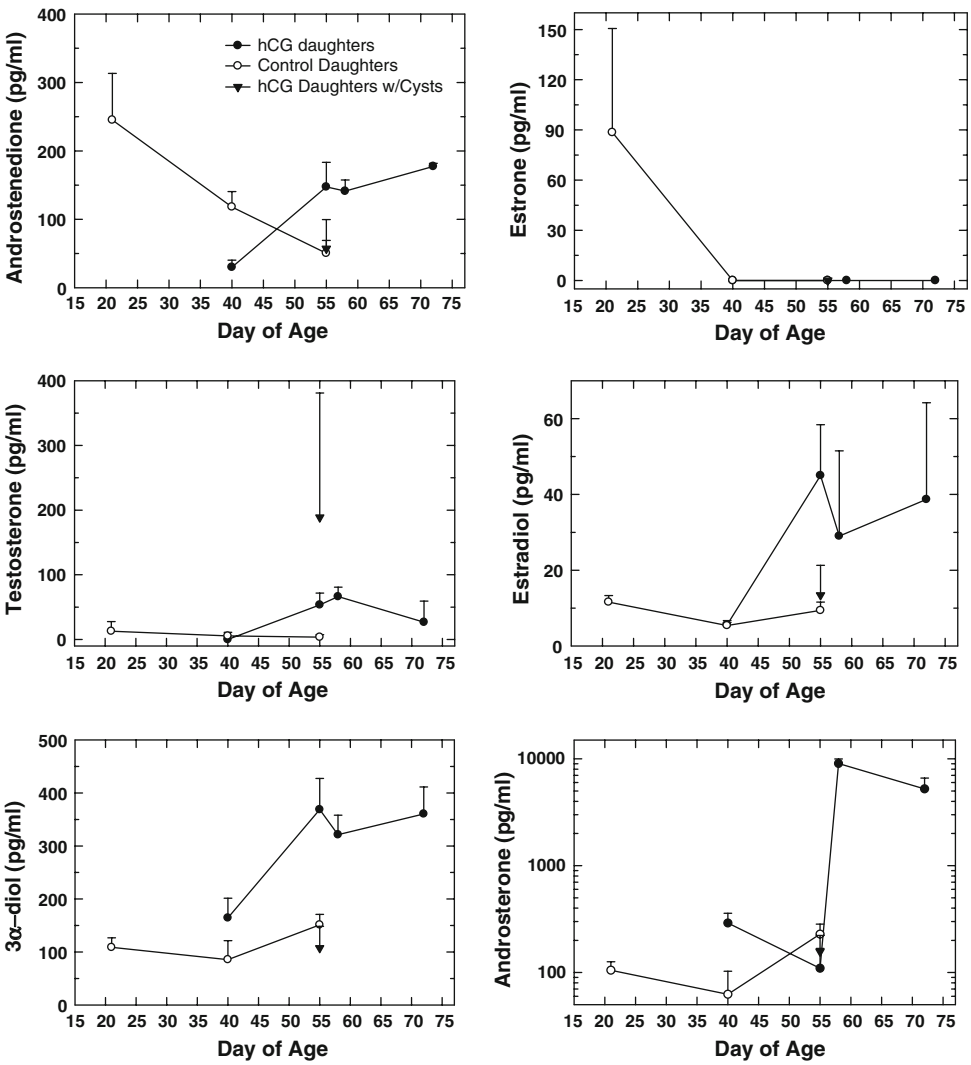

Fig. 5 Serum androgen and estrogen profiles for female progeny from control pregnant rats and 3 IU hCG-treated pregnant rats. Values were obtained from the processed serum samples used to obtain the data in Fig. 4. Data points represent the mean ± SEM

The mean serum testosterone concentration for control daughters was low at 21 days of age (13 pg/ml) and remained low through 55 day of age (3.7 pg/ml) with several rats displaying undetectable amounts of this steroid. In contrast, mean serum testosterone concentrations for *noncystic* daughters from hCG-treated pregnant rats increased from values that were undetectable in our RIA system at 40 days of age to 53 pg/ml by day 55 of age ($P \leq 0.05$). Further, the mean serum testosterone value for these daughters at 72 days of age was highly variable (26.7 ± 32.7 pg/ml), since several of these animals displayed undetectable amounts of serum testosterone. The mean serum testosterone value for the 6 daughters (from hCG-treated pregnant rats) bearing ovarian cysts or precysts also was highly variable (188.7 ± 192.4 pg/ml) on day 55 of age and, therefore, did not differ from values for control daughters or for their noncystic siblings ($P > 0.05$). Further, unlike serum P4 for these 6 animals at 55 days of age, the variability in serum testosterone was not due to the daughter that displayed precystic rather than fully cystic follicles.

Serum E1 concentrations for daughters from control pregnant rats were highly variable (88.5 ± 62.1 pg/ml) at 21 days of age, with several of these daughters displaying undetectable amounts of E1. Further, mean serum E1 concentrations for progeny for both control and 3 IU hCG-treated pregnant rats were undetectable in our RIA system for all other samples tested.

As expected, in contrast to E1, the mean serum E2 concentrations for control daughters were 11.6, 5.4, and 9.4 pg/ml at 21, 40, and 55 days of age, respectively. The mean serum E2 value for *noncystic* daughters of hCG-treated rats was 5.6 ± 0.6 pg/ml on day 40, which was similar to that for daughters from control pregnant rats on this day. However, on day 55, the mean serum E2 value for *noncystic* daughters of hCG-treated rats was 45.0 pg/ml, which was greater than that for control daughters on this day ($P \leq 0.05$). Further, the mean serum E2 values for *noncystic* daughters from hCG-treated rats were maintained at this level on subsequent days tested. However, the highly variable nature of the values probably reflected the asynchronous nature of the spontaneous cycles in these animals. In contrast to 55-day-old *noncystic* daughters from hCG-treated rats, the mean serum E2 value for their *cyst bearing* siblings on this day was 13.4 pg/ml ($P \leq 0.05$) and was similar to the mean serum value observed for daughters of control pregnant rats on this day.

The mean serum 3αdiol concentrations for control daughters were maintained at ~100 pg/ml between 21 and 40 days of age and increased to 151 pg/ml by day 55 of age ($P \leq 0.05$). The mean serum 3αdiol concentrations for *noncystic* daughters from hCG-treated pregnant rats increased from 164 to 367 pg/ml between 40 and 55 days of age ($P \leq 0.05$) and remained at this level on subsequent days tested. The mean value observed on day 55 of age for these animals was greater than the values observed not only for control daughters ($P \leq 0.05$) but also for their 6 *cystic* siblings on that day (107 pg/ml). In contrast, the mean serum value for 3αdiol was similar for the 55 day old daughters from control pregnant rats and the 6 cyst/precyst bearing daughters from hCG-treated pregnant rats.

Serum A5 concentrations for control daughters were maintained at values less than 100 pg/ml between 21 and 40 days of age ($P > 0.05$) and increased to 229 pg/ml ($P \leq 0.05$) by 55 days of age. In contrast, serum A5 values for *noncystic* daughters from hCG-treated pregnant rats decreased from 290 pg/ml ($P \leq 0.05$ compared to controls) on day 40 to 110 pg/ml on day 55 of age ($P \leq 0.05$). The six 55-day-old daughters with *cystic* ovaries displayed a mean serum A5 value of 157 pg/ml, which was not statistically different from either control daughters or their *noncystic* siblings on this day. It is interesting to note that the mean serum A5 concentration for *noncystic* daughters from hCG-treated pregnant rats increased to nanogram amounts and appears to have remained at this level between 58 and 72 days of age ($P \leq 0.05$ compared to all other groups on days 20–55 of age).

Finally, DHT was undetectable in the sera of all pregnant rats and all female progeny from all treatment groups on all days tested (data not shown).

Effect of unabated stimulation by hCG in vivo on maternal serum insulin concentrations.

Figure 6 (left panel) illustrates that maternal serum insulin concentrations were variable for each in vivo treatment group and no differences were observed among the control and hCG-treated groups on any day tested.

The expected peripubertal increase in serum insulin occurred between days 20 and 55 of age for daughters from control pregnant rats. No statistical differences in mean serum insulin were observed when values for daughters from control pregnant rats were compared with values for daughters from 3 IU hCG-treated pregnant rats (with or without cystic ovaries) on days 40 and 55 of age, respectively ($P > 0.05$).

Discussion

This series of experiments demonstrates, for the first time, that the maternal ovarian cystic state induced by unabated stimulation with subovulatory doses of hCG can trigger the development of ovarian cysts shortly after the window of puberty in a cohort of the resulting female progeny. This spontaneous induction of ovarian cysts in the daughters of

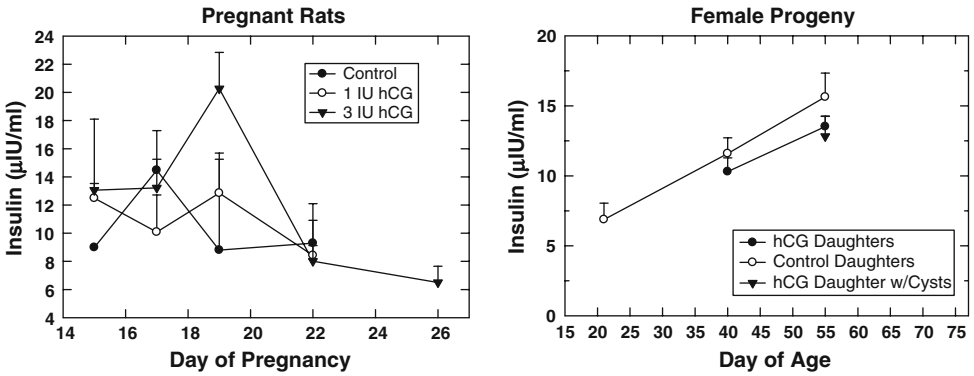

Fig. 6 Serum insulin profiles for control pregnant rats and pregnant rats treated with increasing, subovulatory doses of hCG and for daughters of control and 3 IU hCG-treated pregnant rats. Values were obtained using an aliquot of serum reserved from the samples used to obtain serum steroid profiles. Serum samples were obtained as described in "Materials and methods." Data points represent the mean ± SEM

hCG-treated pregnant rats, in contrast to the complete lack of ovarian cysts in age-matched daughters from control pregnant rats, occurs in the *absence* of an endogenous, maternally sustained, hyperandrogenic or hyperinsulinemic state and in the *presence* of tonically elevated circulating estrogens. These results support our recent observations in HYPOXD rats that estrogens, rather than androgens, play a crucial role in the induction of ovarian cysts [19].

The inability to detect DHT in the maternal peripheral serum was not unexpected since it is well established that P4 is the preferred substrate for 5α-reductase in both rodent and human ovaries, which metabolize P4 efficiently to 5α-reduced C_{21}-steroids, which are then metabolized to A5 and 3αdiol [28–30]. This preference for progestins as substrate, rather than androgens, by both rodent and human ovarian 5α-reductase appears to protect at least luteal P4 production from the suppressive effects of DHT [31]. Small antral and preovulatory follicles [32], developing ovarian cysts [16], and cultured thecal shells from small antral and preovulatory follicles [32] also have a limited capacity to produce DHT in vitro even in the presence of exogenous A4 or testosterone. Together, these observations and the observation that serum A5 parallels serum P4 profiles for each in vivo treatment group indicates that the corpora lutea are the primary source of circulating A5 in both the control and hCG-treated pregnant rats.

The source of the sustained increases in circulating E2 and E1 during the induction of ovarian cysts in pregnant rats with 3 IU hCG is not certain at this time since short-term administration of this dose of hCG to pregnant rats in mid-pregnancy not only stimulates *follicular* steroidogenesis [33] but also the ability of *corpora lutea* to produce testosterone and E2 [34]. Developing ovarian cysts in hCG-treated pregnant rats [15, 16] and in HYPOXD rats treated with FSH+hCG [18] display the capacity to produce preovulatory amounts of E2 in the presence of a kinetically saturating amount of either A4 or testosterone in vitro [15, 16, 18]. Further, in FSH+hCG-treated HYPOXD rats, circulating concentrations of A4 and E2, but not E1, parallel the induction of large ovarian cysts [18]. Thus, the changes in circulating E1 in response to 3 IU hCG in the present series of experiments appear to reflect increased luteal metabolism of A4 to E1, whereas the changes in circulating E2 appear to reflect changes in follicular steroidogenesis that accompany the induction of ovarian cysts by subovulatory amounts of hCG.

Although large amounts of exogenous 5α-reduced androgens can modulate the kinetics of the aromatase reaction in granulosa cells from preovulatory follicles in vitro [35], the combined effects of endogenously produced testosterone, 3αdiol, and A5 were not sufficient to suppress the increase in serum estrogens and, by extension, estrogen production in hCG-treated pregnant rats. Indeed, the steroid profiles observed during the induction of precystic follicles in pregnant rats treated with as little as 1 IU hCG underscore the concept that, at least in rats, hyperandrogenism is not required to trigger the ovarian mechanisms involved in the development of follicular cysts [19]. These observations, the ability of diethylstilbestrol (DES) to induce hyper-responsive neonatal mouse ovaries in vivo and in vitro [36], the inability of DHT to induce an ovarian cystic state in the progeny of androgenized ewes [10], and the observation that E2 plays an important role in regulating the number of follicles in the inner and outer cortical regions of the fetal baboon ovary during mid to late pregnancy [37] support the concept that prolonged exposure to sustained increases in estrogen may play a more crucial role than hyperandrogenism in the induction of ovarian follicular cysts.

Although one can never directly extrapolate concepts obtained in nonhuman mammalian models to the human situation, it is interesting to note that one of the most

common problems encountered during treatment of infertility in association with PCOS is the hyperstimulated response of the ovaries to the hormonal treatment regimens used in assisted reproduction programs. The concept that the ovary can respond to hormonal stimuli during pregnancy with either normal or abnormal follicular development is supported by our present observations, the ability of ovaries in pregnant rats to respond to subovulatory doses of hCG with increased follicular and luteal steroidogenic ability [15, 16, 33, 34], the correlation between circulating testosterone concentrations in pregnant Rhesus monkeys with circulating amounts of chorionic gonadotropin [38, 39], and the ability of human ovaries to respond to exogenous gonadotropin at least during early pregnancy with ovarian follicular development and even ovulation [40]. On the basis of these observations, and the observation that many women with PCOS possess hyperstimulable ovaries [41], it is conceivable that human ovaries that are "hyperstimulable" prior to pregnancy may respond to nine months of tonic exposure to endogenous hCG with inappropriate follicular, luteal and, perhaps even placental steroidogenic profiles that contribute to the etiology of ovarian cysts and possibly PCOS in affected daughters.

This concept does not exclude a role for androgens in the development of ovarian cysts or in the establishment of a neuroendocrine environment that contributes to the establishment of an oligomenorrheic, cystic-ovary state. First, sufficient aromatizable androgen must be available to support the amounts of circulating estrogens observed in the present study and in FSH+hCG-treated HYPOXD rats [18], and to support the mid-follicular phase levels of E2 typically observed in women with PCOS [1–3]. Second, although combined tonic stimulation by FSH plus E2 is required for the induction of polyfollicular ovarian cysts in HYPOXD rats [19], "pure" androgen-like activity in addition to stimulation by FSH+E2 is required for the development of a "string of pearls" ovarian "architecture" [19] that mimics the gross ovarian morphology characteristic of women with PCOS [1–3]. Third, endogenous estrogens can have local positive or negative feedback effects on ovarian thecal androgen production [42–46] and, thereby, can modulate the specific positive and negative impact that androgens can have on different stages of follicular development.

Although proximity to males in utero affects testosterone values in the amniotic fluid and blood of female *mouse* fetuses and correlates with a postnatal impact on the length of the estrous cycle and the secondary sex characteristics of the female offspring [47–51], this effect does not appear to occur in rats or the ICR mouse [52, 53], or in our present model. Still, the spontaneous development of ovarian cysts and precysts in 24% of 55-day-old daughters of pregnant rats treated with 3 IU hCG suggests that females positioned between two males in utero may be more susceptible to the impact of prolonged, moderate increases in the maternal estrogenic environment than other female offspring. Thus, *combined*, tonic exposure to inappropriately elevated local estrogens and androgens in utero may be required to trigger neuroendocrine and ovarian epigenetic responses that result in the spontaneous expression of cystic ovaries in this cohort of female offspring.

In distinct contrast to the daughters of hyperandrogenized sheep and monkeys [10, 11], neither hCG-treated pregnant rats nor their daughters develop hyperinsulinemic states. Although the reasons for this are not clear at this time, this observation clearly indicates that the mechanisms involved in the establishment of a hyperinsulinemic state in the daughters of pregnant sheep [10] and monkeys [11] by exposure to excess amounts of testosterone propionate in utero, can be distinguished empirically from the mechanistic pathway that triggers the peripubertal expression of ovarian cysts in a susceptible cohort of daughters.

This is an important observation since ~44% of women diagnosed as having PCOS and participating in the national study conducted by Warner-Lambert/Park-Davis (presented by Andrea Dunaif at the Annual Meeting of the Endocrine Society, Minneapolis, MN) do not display insulin resistance/hyperinsulinemia. Further, up to 26% of normal girls and adolescent women—with no indicators of either insulin resistance or other indicators of susceptibility to PCOS—spontaneously express polycystic ovaries [54–56] in a manner that correlates positively with age [54]. Thus, our observations not only provide support for the concept of a role for a maternal estrogenic environment in triggering the spontaneous development of ovarian cysts in female progeny, but also underscore the fact that the spontaneous development of cystic ovaries in both rodents and women does not require a hyperinsulinemic/insulin resistant state.

It is interesting to note that estrogen-binding proteins are present in the pancreas of at least rodents and primates [57–61], and are thought to have an important role in maintaining normal pancreatic function. These binding proteins display high specificity for estrogens, and can bind conjugated weak androgens (which are elevated in women with PCOS [62]), but have virtually no affinity for strong androgens [61]. Thus, it is possible that the normal role for estrogen in the pancreas is intact in at least the young daughters of pregnant rats treated with 3 IU hCG. Whether or not this is the case in these animals as they become older and display increasing concentrations of serum 5α-reduced androgens, or in the pancreatic tissues of androgenized ewes [10] and monkeys [11] remains to be determined.

An important hallmark of the transition from prepubertal to adult ovarian function, in at least rodents, is the marked

switch from the preferential metabolism of P4 to 5α-reduced C_{19}-steroids to the preferential metabolism of P4 to aromatizable androgens, such as A4 and testosterone [28, 63–65]. Whether or not the striking age-related increase in serum concentrations of 3αdiol and A5 for postpubertal noncystic daughters of pregnant rats treated with 3 IU hCG, presented here, is the result of preferred metabolism of P4 to 5α-progestins and thence to 5α-reduced androgens [28, 63–65] remains to be determined. Indeed, the present observations raise an intriguing question regarding the preferred substrate for 5α-reductase in ovarian follicles and cysts of women with PCOS, since it is well established that circulating progestins (but not P4) are elevated in these individuals (1–3).

In rodents, elevated, prepubertal serum concentrations of 3αdiol and A5 suppress normal ovarian follicular development and E2 production, as well as gonadotropin secretion and the ovulatory gonadotropin surge [28, 63–65]. Therefore, the observation of an aberrant, marked increases in postpubertal circulating A4, 3α-diol, and A5 and even E2 are of special interest because these steroids, unlike testosterone, seem to be consistently elevated in individuals with PCOS compared to early follicular phase serum concentrations of these steroids in normally cycling women [1–3, 62]. Thus, it is tempting to speculate that exposure in utero to a maternal cystic ovary state may result in epigenetic imprinting that results in hyperstimulable ovaries in affected daughters. The postpubertal functional transition that appears to be occurring in the noncyst-bearing daughters of pregnant rats treated with 3 IU hCG may be a hallmark of such hyperstimulable ovaries. Whether or not this transition will lead to a progressively worsening situation in these animals, as appears to occur in many individuals that express PCOS [1–3], remains to be determined. However, the observation that the daughters of hyperandrogenized pregnant ewes appear to undergo an age-related transition to a polycystic ovarian state in which a cohort of daughters is affected at a younger age than the majority of androgenized daughters [10, 66] provides indirect support for the notion that a similar situation may be developing in these older daughters from cyst-bearing pregnant rats.

In conclusion, our present observations not only support the general concept that exposure to an aberrant maternal steroid environment can trigger abnormal reproductive development in utero [10, 11, 20–27, 66], but also underscores the concept that exposure to a factor other than excess androgens or hyperinsulinemia is crucial for the spontaneous induction of ovarian cysts in affected female progeny. Thus, the daughters that express ovarian cysts at 55 days of age may reflect the impact of combined, tonic exposure to increased estrogens (E2 and E1) and local endogenous androgens due to positioning between male siblings in utero. In contrast, the development of abnormal 5α-reduced androgen profiles in the older, as yet noncystic daughters appears to reflect the impact of tonic exposure to increased estrogens and 5α-reduced androgens but limited exposure to endogenous androgens produced by male siblings in utero. Whether or not the observed increases in serum 3αdiol and A5 precede the induction of ovarian cysts in these older daughters of hCG-treated pregnant rats remains to be determined. These observations, together with those regarding the daughters of hyperandrogenized pregnant sheep [10, 66] and monkeys [11] strongly support the exciting concept that inappropriate stimulation by estrogens in utero may play a crucial role in triggering the spontaneous development of an ovarian cystic state in both polyovular and monovular mammals.

Materials and methods

Materials

Human chorionic gonadotropin (4,000 IU/mg) and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO). Medium 199 was obtained from Grand Island Biological Co. (Grand Island, NY). All nonradioactive steroids were obtained from Steraloids Inc. (Wilton, NH). Tritiated steroids were obtained from DuPont New England Nuclear (Boston, MA). Iodinated E2 and A4 were obtained from Diagnostic Systems Laboratories (Webster, TX). Iodinated P4 and testosterone as well as insulin assay kits were obtained from Diagnostic Products Corporation (Los Angeles, CA). Iodinated E1 and its antisera were obtained from Pantex (Santa Monica, CA). Antisera for testosterone was the generous gift of Dr. Gerry Nordblom (Ann Arbor, MI). Antisera against A4 was generously provided by Drs. Gerry Nordblom and Barry England (Ann Arbor, MI). Antisera against E2 and P4 were generously provided by Dr. Gordon Niswender (Fort Collins, CO). Antisera against androsterone was generously provided by Endocrine Sciences (Tarzana, CA). Antisera for DHT and 5α-androstane-3α,17β-diol (3αdiol) were obtained from Dr. P.N. Rao (San Antonio, TX).

Animals and histology

First-time pregnant rats (approximately 200 g), with the day of mating designated as day 1 of pregnancy, were obtained from Harlan Sprague–Dawley (Indianapolis, IN) and housed in the University of South Carolina School of Medicine Animal Resources Facility. Animals were treated and handled according to a research protocol approved by the USC Animal Use Committee. The animals were housed in plastic containers containing aspen in a temperature-controlled

room with a light:dark cycle of 12:12. Beginning on day 13 of pregnancy and continuing for 9 days, these animals received twice daily subcutaneous injections of either 0 (controls), 1, or 3 IU hCG in 0.2 ml 10 mM phosphate buffered saline containing 0.09% pig skin gelatin, pH 7.0 (gel-PBS) as previously described [15].

Between 0800 and 0900 h on the days indicated in the "Results" section, a total of 6–13 pregnant rats from each treatment group were decapitated, trunk blood was collected in polypropylene tubes for each rat, and ovaries were excised. Blood samples were centrifuged at 4°C and individual serum samples were aliquoted into separate polypropylene tubes and stored at −20°C. Ovaries from each treatment group were fixed in formalin for sectioning and hematoxylin–eosin staining by the Histology Core Facility of the USC School of Medicine. Ovarian sections were examined using a Zeiss 35IM microscope.

In order to determine the effects of the in vivo hormonal treatments on the outcome of pregnancy and on the reproductive development of the progeny, a number of control and hCG-treated pregnant rats were permitted to undergo parturition. Pups were decapitated between 0800 and 0900 h on the days of age indicated in the "Results" section. The number of 20-, 40-, and 55-day-old daughters used from control pregnants was 4, 17, and 17, respectively. The number of 40- 55- 58- and 72 day-old daughters used from pregnant rats treated with 3 IU hCG was 15, 25, 11, and 3, respectively. Trunk blood was collected in polypropylene tubes for each pup, and ovaries or testes from the pups were dissected free of adhering connective tissue and fixed in formalin, for sectioning and hematoxylin-eosin staining by the Histology Core Facility of the USC School of Medicine. Blood samples were processed in the same manner as described for maternal blood. Gonadal sections were examined using a Zeiss 35IM microscope.

Radioimmunoassays (RIAs)

In order to determine serum steroid concentrations, known volumes of gel-PBS or serum sample (each containing a verified amount ($\sim$1,500 cpm) of the tritiated steroids of interest to monitor for procedural recoveries) were extracted individually with diethyl ether and separated by HPLC using previously described procedures that are routinely used in this laboratory [15–18]. Briefly, sample aliquots were extracted individually with diethyl ether; the ether phases were evaporated; and the residues were dissolved in AcN:H_2O (40:60). The reconstituted samples were loaded onto a Rainin C_{18} microsorb column, the steroids were separated using a Rainin Rabbit HPLC Gradient system, and column effluent was collected using a Gilson FC-80 fraction collector. Effluent fractions associated with the elution of the specific steroids of interest were evaporated, reconstituted with 1 ml gel-PBS, and stored at −20°C until analyzed by RIA. Procedural recoveries were assessed by counting a precise volume (0.2 ml) of each of the reconstituted samples in a Beckman LS150 or a Packard Tricarb scintillation counter and determining the fraction of each tritiated steroid recovered in its appropriate reconstituted sample. The remaining portion of each reconstituted sample was analyzed by RIA for the appropriate steroid of interest.

Steroid RIAs were performed according to previously established procedures [15–19, 32, 33]. In our laboratory, RIAs for testosterone, DHT, A4, A5, and 3αdiol use standard curves that range from 4 to 1,000 pg. The E1 assay uses a standard curve that ranges from 0.4 to 100 pg, while the E2 assay uses a standard curve that ranges from 0.1 to 25 pg. The lowest point for each of these standard curves is greater than the limit of detection (reference binding ± 2SD) for its respective assay. Intra- and inter-assay coefficients of variation for the steroid RIAs were: 3αdiol (10% and 15%), androstenedione (6% and 18%), androsterone (15% and 13%), DHT (8% and 17%), estradiol (6% and 10%), estrone (7% and 12%), progesterone (9% and 15%), and testosterone (4% and 10%).

Insulin assays were performed according to the manufacturer instructions included with the kits. Intra- and inter-assay coefficients of variation for the insulin assay were 7 and 10%, respectively.

Statistics

Statistical differences between and among treatment groups for individual steroids and for insulin were determined by multiple comparison analysis of variance and Student–Newman–Keuls test to isolate significant differences. Differences were considered significant when $P \leq 0.05$.

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