Endocr (2007) 31:179–192
DOI 10.1007/s12020-007-0028-5

ORIGINAL PAPER

Obligatory roles for follicle-stimulating hormone (FSH), estradiol and androgens in the induction of small polyfollicular ovarian cysts in hypophysectomized immature rats

Katryna Bogovich

Received: 12 January 2007 / Accepted: 5 April 2007 / Published online: 10 July 2007

Abstract Immature hypophysectomized (HYPOXD) rats develop large, polyfollicular ovarian cysts in response to unabated, combined stimulation by subovulatory doses of human chorionic gonadotropin (hCG) and highly purified ovine follicle-stimulating hormone (FSH). Further, circulating amounts of androstenedione (A4) and estradiol (E2), but *not* testosterone or dihydrotestosterone (DHT), change in parallel with the development of these cysts. To determine the potential roles of either A4 or E2 at the level of the ovary in the induction of ovarian cysts, pellets containing either (1) cholesterol (placebo; controls); (2) A4; or (3) E2 were administered subcutaneously (sc) to immature HYPOXD rats. Some of these animals also received either twice-daily sc injections of 1 IU hCG, or daily sc injections of 2 μg FSH, for 13 days. Ovaries and sera were harvested from all treatment groups on the morning of day 14 of the combined-hormone treatment schedule. As expected, ovaries from HYPOXD rats treated with placebo, A4, or E2 pellets (with or without hCG) failed to display antral follicles. Ovaries from HYPOXD rats treated with FSH and a placebo pellet displayed polyfollicular, atretic, small antral follicles with unstimulated thecal shells. In addition, the ovarian stromal-interstitial tissue had an unstimulated appearance. In contrast, ovaries from HYPOXD rats treated with FSH plus either A4 or E2 implants displayed stimulated stromal-interstitial tissue as well as small follicular cysts and precysts with stimulated thecal shells. The number of cysts and precysts observed in the largest ovarian cross-sections for animals treated with FSH + A4 (17.0 ± 3.0) was less than that observed in the largest ovarian cross-sections for HYPOXD rats treated with FSH + E2 (40.2 ± 10.1; $p < 0.05$). To determine if the development of ovarian cysts in response to FSH + A4 was due, at least in part, to the metabolism of A4 to E2, HYPOXD rats were treated with either (1) placebo pellets; (2) pellets containing dihydrotestosterone (DHT) which cannot be metabolized to estrogen; (3) E2 pellets plus DHT pellets (E2 + DHT); (4) FSH + DHT; or (5) FSH + E2 + DHT. The largest ovarian cross-sections from FSH + DHT-treated HYPOXD rats displayed 18.3 ± 4.1 small follicles with a mean diameter of ~0.437 mm which possessed few granulosa cells. The thecal and stromal-interstitial tissues in these ovaries were unstimulated, which indicates that these small degenerating follicles were atretic rather than cystic. In contrast, the largest ovarian cross-sections from FSH + E2 + DHT-treated HYPOXD rats displayed 51.6 ± 2.4 cysts with stimulated thecal shells and a mean diameter of ~0.634 mm. Further, these cysts were arranged in a "string of pearls" pattern and the ovarian stromal-interstitial tissue possessed a stimulated appearance. These data demonstrate a direct, unambiguous role at the level of the ovary for unabated tonic stimulation by FSH plus estrogen in the development of *small* polyfollicular cysts in HYPOXD rats. Further, the data also indicate that, at least in HYPOXD rats, combined, tonic stimulation by FSH plus estrogen and androgen is sufficient for the development of small, polyfollicular ovarian cysts in a "string of pearls" pattern. These observations are in distinct contrast to our previous observations that tonic stimulation by FSH + hCG results in the induction of *large* ovarian cysts in HYPOXD rats and provide tantalizing new insights regarding the potential importance of specific hormones at the level of the ovary in the induction of specific types of cystic follicles.

K. Bogovich (✉)
Department of Obstetrics & Gynecology, University of South Carolina School of Medicine, Building 28, First Floor, Columbia, SC 29209, USA
e-mail: bogovich@gw.med.sc.edu

Keywords Polycystic ovaries · Ovarian cysts · Hypophysectomized rats · Estrogen action · Androgen action · FSH · PCO · PCOS

Introduction

Ovarian cysts develop spontaneously in many mammalian species, including but not limited to cows, dogs, pigs, rats, cats, and women [1–17]. Unlike the synchronized hormonal profiles associated with normal reproductive cycles, the peripheral hormonal environments associated with established cystic ovary states are extremely heterogeneous and display only sporadic cyclicity. By definition the peripheral hormonal environment associated with an already established cystic ovary state cannot identify the specific factors involved in the induction of that state in a heterogeneous system. This conceptual dilemma has delayed the development of consistently reproducible models that can elucidate, unambiguously, the specific factors and fundamental mechanisms involved in the induction of cystic follicles in the mammalian ovary.

The ovaries of immature, adult, and pregnant rats, whose intact neuroendocrine units have been suppressed, consistently respond to unabated stimulation by subovulatory doses of hCG with the synchronized development of a physiologic number of large ovarian follicular cysts (3–5 mm in diameter) [18–20]. These observations were supported by the results obtained by other investigators in subsequent studies that used other mammalian species such as: mice (in which LH was transiently overexpressed) [21]; the estrogen receptor knockout mouse (in which negative and positive neuroendocrine feedback mechanisms are disrupted) [22]; cattle (in which the gonadotropin surge was blocked) [23]; and gilts and sows [24, 25].

Since the number of bilateral ovarian cysts induced by hCG in intact, yet neuroendocrinologically suppressed rats [18–20] equals the number of preovulatory follicles normally observed in this species, these ovaries should be referred to as "*cystic*" rather than "polycystic". In contrast, exposure of immature HYPOXD rats to combined, unabated stimulation by hCG and highly purified FSH consistently results in the synchronized induction of a supraphysiologic number of large ovarian cysts [26]. Therefore, these ovaries should be referred to as "*polycystic*".

Peripheral serum concentrations of androstenedione (A4) and estradiol (E2), but *not* testosterone or DHT, rise and fall in the FSH + hCG-treated HYPOXD rat in a manner that parallels the induction of these large polyfollicular ovarian cysts [26]. This may be an extremely important observation since total circulating A4, dehydroepiandrosterone (DHEA), E2, and estrone (E1) are consistently elevated in at least women with PCOS [15–17]. In contrast, *total* circulating testosterone and DHT generally are not consistently elevated in these subjects unless a secondary endocrinopathy is present [15–17]. Together, these observations raise questions about the relative roles of tonic stimulation by androgens and estrogens, at the level of the ovary, in the induction of cystic follicles.

The immature HYPOXD female rat provides a highly controlled in vivo environment that can be used not only to delineate the specific roles of FSH and E2 in normal ovarian follicular development [27, 28], but also to investigate the relative roles of FSH and LH-like activity in the induction of large polyfollicular ovarian cysts [26, 29]. Therefore, the immature HYPOXD female rat was used in the present series of experiments to investigate the potential roles, at the level of the ovary, of tonic exposure to androgens and/or E2 in the induction of cystic follicles.

Results

As in our previous studies, the term "cystic follicles" refers to follicles that possess thecal shells with a stimulated appearance and just a remnant of granulosa cells [18–20, 26, 29]. "Precystic follicles" are similar in appearance to small cystic follicles, but have not yet lost as many granulosa cells as fully cystic follicles. Neither cystic nor precystic follicles display signs of involution. In contrast, although "atretic follicles" display lost of granulosa cells, they do not possess stimulated thecal shells and often show signs of involution.

Impact of hormonal treatments on serum steroid concentrations

Serum steroid concentrations at the end of the in vivo treatment regimen are shown in Table 1. Serum from control HYPOXD rats bearing placebo (1.5 or 5.0 mg cholesterol) pellets did not display detectable amounts of any steroid of interest on the morning of the last day of the in vivo treatments. In contrast, sera from all HYPOXD rats that were given A4, E2, or DHT pellets displayed detectable amounts of their respective implanted steroids on the morning after the last day of the in vivo treatments. It is not known at this time if the relatively low values observed in all DHT-pelleted groups were due to the dynamics by which the pellets secreted DHT or to an extremely efficient metabolism of DHT to other, less potent, 5α-reduced entities.

The data in Table 1 indicate that, by the last day of the in vivo treatment regimens, the steroids secreted by the implanted pellets did have an effect on the concentrations

Table 1 Serum steroid concentrations at the end of the in vivo treatments for animals bearing the indicated pellets

Steroid pellet	A4 (ng/ml)	Testosterone (ng/ml)	E2 (ng/ml)	E1 (ng/ml)	DHT (ng/ml)
Placebo (cholesterol)	ud[a]	ud	ud	ud	ud
1.5 mg A4	1.4 ± 0.29	0.92 ± 0.11	ud	ud	ud
5 mg A4	3.0 ± 0.40	2.3 ± 0.33	ud	0.021 ± 0.006	ud
1.5 mg E2	0.34 ± 0.21	0.057 ± 0.064	1.5 ± 0.56	0.210 ± 0.023	ud
5.0 mg E2	0.41 ± 0.27	0.180 ± 0.079	6.3 ± 0.81	0.880 ± 0.150	ud
1.5 mg DHT	0.14 ± 0.039	ud	0.003 ± 0.002	ud	0.176 ± 0.037
5.0 mg DHT	0.18 ± 0.062	0.24 ± 0.048	0.006 ± 0.004		0.501 ± 0.085

[a] ud: undetectable

of some of the other steroids observed in the sera of these animals. Thus, sera from HYPOXD rats bearing A4 pellets displayed detectable amounts of testosterone, but not E2 or DHT by the last day of the in vivo treatments. Sera from HYPOXD rats bearing E2 pellets displayed detectable amounts of A4, testosterone, and E1 but not DHT by the last day of the in vivo treatments; and sera from animals bearing DHT pellets displayed detectable amounts of A4, testosterone, and E2 but not E1. Neither hCG nor FSH had a sustained effect on peripheral serum steroid concentrations that could be detected by day 14 either alone or in the presence of either placebo- or steroid-pellets in HYPOXD rats. Finally, combined treatment of HYPOXD rats with DHT and E2 pellets, in the presence or absence of gonadotropin, resulted in serum steroid concentrations on the last day of the in vivo treatments that were additive when compared to the serum steroid concentrations observed in similar rats treated with either E2 or DHT alone.

Impact of hormonal treatments on ovarian morphology

To simplify the presentation of the data from these experiments, whenever there was no significant difference between the results obtained using a lower dose and a higher dose of steroid in a particular treatment regimen only the results obtained with the higher dose of steroid were depicted in the figures.

Ovaries from HYPOXD rats bearing placebo, A4, E2, or both A4 and E2 pellets were small (Fig. 1A–D, respectively) and possessed only preantral follicles. It is interesting to note that the thecal shells in the ovaries from HYPOXD rats bearing A4 + E2 pellets (Fig. 1D) possessed a more stimulated appearance than the thecal shells in the ovaries from placebo-, A4-, or E2-pelleted HYPOXD rats (Fig. 1A–C, respectively).

Figure 2 illustrates that there was no difference between the largest cross-sectional areas (LCAs) of ovaries from HYPOXD rats bearing placebo pellets and similar rats

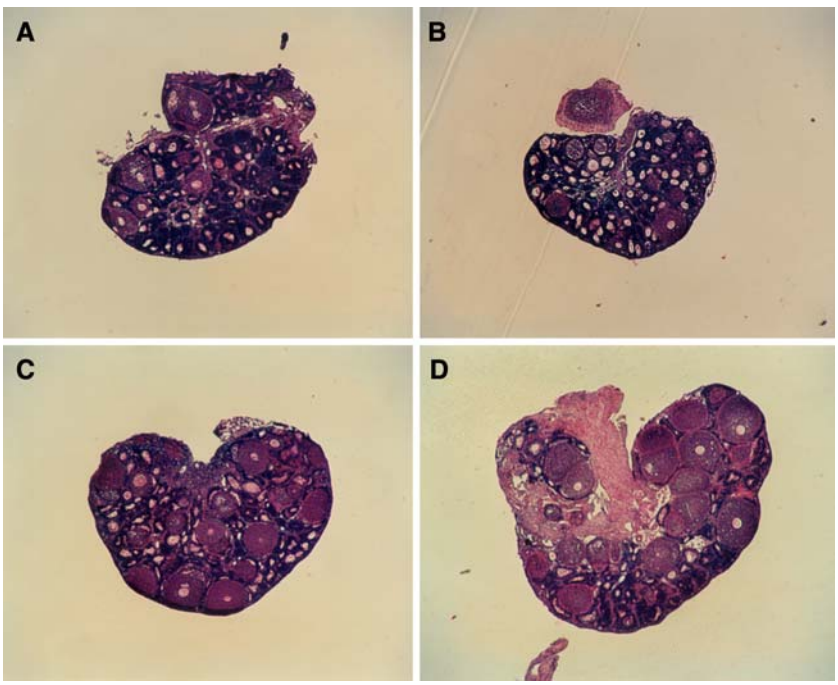

Fig. 1 Impact of placebo-, A4-, and E2-pellets on ovarian morphology of the HYPOXD rat. Ovarian cross-sections from (**A**) control HYPOXD rats (placebo pellets); and from HYPOXD rats treated with (**B**) 5 mg A4 pellets; (**C**) 5 mg E2 pellets; or (**D**) both A4 (5 mg) and E2 (5 mg) pellets. Photomicrographs were obtained on a Zeiss IM35 microscope using a 2.5× objective

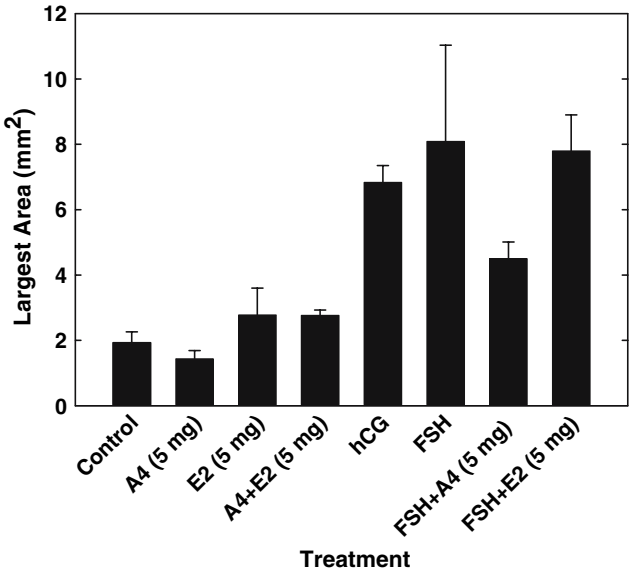

Fig. 2 Impact of A4, E2, hCG, and FSH on the largest cross-sectional areas (LCAs) of ovaries from HYPOXD rats. Each bar represents the mean ± SEM for ovaries from each treatment group from at least two experiments as described in the ''Materials and methods.'' Significant differences ($p \leq 0.05$) between groups are described in the text

bearing pellets containing either 5.0 mg of A4 or E2 (1.93 ± 0.33, 1.44 ± 0.25 and 2.78 ± 0.82 mm^2, respectively). Further, the LCAs did not change in response to increasing doses of steroid for either A4 or E2 (data not shown). Figure 2 also illustrates that the mean value of the LCAs for ovaries from animals bearing only E2 pellets was almost identical to that of ovaries from HYPOXD rats bearing A4 + E2 pellets (2.77 ± 0.16 mm^2). However, the increased uniformity in the size of the ovaries from A4 + E2-pelleted HYPOXD rats, compared to ovaries from similar animals bearing only E2 pellets, resulted in a statistically larger mean LCA ($p \leq 0.05$) for A4 + E2-pelleted HYPOXD rats than that observed for placebo- or A4-pelleted HYPOXD rats.

As noted previously [26, 29], the largest follicles present in the ovaries of HYPOXD rats treated with hCG alone were at the preantral stage of development (histology not shown here). Figure 3 illustrates that the largest follicles in the ovaries of HYPOXD rats treated with hCG plus either A4 or E2 (Fig. 3A and B, respectively) also were at the preantral stage of development. Further, the LCAs for ovaries from hCG-treated and from hCG + steriod-treated HYPOXD rats were similar. Therefore, only the mean LCA for ovaries from hCG-treated HYPOXD rats has been presented in Fig. 2. As in our previous work with this model [26, 29], the absence of antral follicles in the ovaries of animals treated with hCG ± steriod indicates that this preparation of hCG was not contaminated with significant amounts of human FSH.

By the morning of day 14 of the combined-hormonal treatments, HYPOXD rats treated with FSH alone possessed ovaries that were very similar in appearance to those observed in our earlier studies with HYPOXD rats [26, 29; histology not shown here]. These ovaries displayed large numbers of atretic, small antral follicles with unstimulated thecal and stromal-interstitial tissues. In contrast, ovaries from both FSH + A4- and FSH + E2-treated HYPOXD rats (Fig. 3C and D, respectively) displayed precystic and

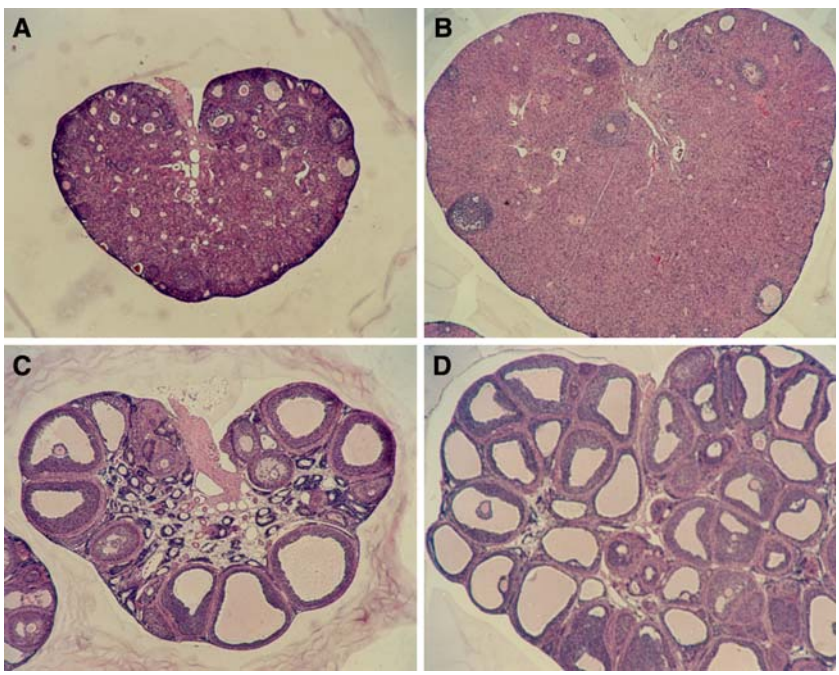

Fig. 3 Impact of combined treatment with gonadotropins and either A4 or E2 on ovarian morphology of the HYPOXD rat. Ovarian cross-sections from rats treated twice-daily for 13 days with 1 IU hCG in the presence of a 5 mg pellet of (**A**) A4; or (**B**) E2; and from rats treated with daily injections of 2 μg oFSH in the presence of a 5 mg pellet of (**C**) A4; or (**D**) E2. Photomicrographs were obtained using a Zeiss IM35 microscope with a 2.5× objective

cystic follicles by the morning of day 14 of the combined in vivo treatments. In contrast to ovarian tissue from HYPOXD rats treated with FSH alone [26, 29], both thecal shells and stromal-interstitial tissue in the ovaries from the FSH + steroid-treated groups possessed a stimulated appearance.

Figure 2 illustrates that the mean LCAs for ovaries from HYPOXD rats treated with either hCG or FSH alone were significantly larger (6.84 ± 0.51 and 8.09 ± 2.94 mm^2, respectively) than the LCAs of ovaries from placebo-pelleted or steroid-pelleted HYPOXD rats ($p < 0.05$). The mean LCA for the ovaries from FSH-treated HYPOXD rats was not statistically different from the mean LCAs for the ovaries from FSH + A4- and FSH + E2-treated HYPOXD rats due to variability in the size of the ovaries from the FSH-only treated animals. However, as shown in Fig. 2, the mean LCA for ovaries from FSH + A4-treated animals (4.5 ± 0.51 mm^2) was smaller ($p \leq 0.05$) than that for ovaries from FSH + E2-treated animals (7.8 ± 1.1 mm^2).

Figure 4 illustrates the impact of the in vivo hormonal treatments on ovarian follicular diameters. The largest preantral follicles in ovaries from placebo-, A4-, and hCG-treated HYPOXD rats displayed similar diameters (0.28 ± 0.01, 0.30 ± 0.09, and 0.32 ± 0.02 mm, respectively) which were smaller than ($p \leq 0.05$) the diameters of the largest preantral follicles in ovaries from E2-, E2 + A4- and E2 + hCG-treated HYPOXD rats (0.36 ± 0.05, 0.36 ± 0.03, and 0.36 ± 0.03 mm, respectively, $p \leq 0.05$).

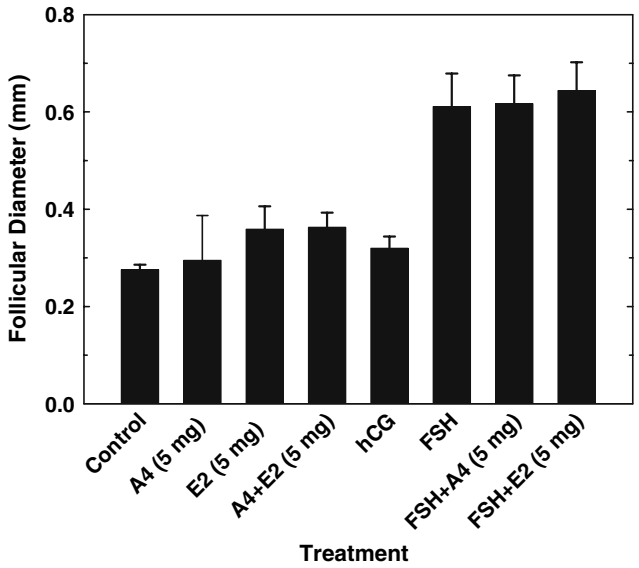

Fig. 4 Impact of prolonged hormonal treatments on the diameters of ovarian follicles in the HYPOXD rat. Each bar represents the mean ± SEM for the average, largest diameter for the 4–6 largest follicles present in the ovaries of each rat in the indicated treatment groups. These values were obtained using the ovarian histological sections used to calculate LACs in Figs. 1 and 3. Significant differences ($p \leq 0.05$) between groups are described in the text

Further, as expected, the diameters of the largest antral follicles in the ovaries of FSH-, FSH + A4-, and FSH + E2-treated HYPOXD rats (0.61 ± 0.07, 0.62 ± 0.06, and 0.64 ± 0.06 mm, respectively) were greater than the diameters of the preantral follicles in the ovaries of any in vivo treatment group that did not receive FSH ($p \leq 0.05$).

Impact of DHT, E2, and FSH on ovarian morphology

A4 can be metabolized in the follicle to testosterone, and testosterone can be metabolized to E2 and/or 5α-reduced non-aromatizable androgens such as DHT [30]. Therefore, it was important to determine if the ability of FSH + A4 to drive cyst development in the ovaries of HYPOXD rats was due to androgen and/or estrogen action. To this end, HYPOXD rats were treated with 1.5 mg or 5.0 mg DHT pellets either alone or in combination with E2 pellets, with or without daily injections of FSH.

Figure 5A and B, respectively illustrate that the largest follicles present in the ovaries of DHT- and DHT + E2-treated HYPOXD rats were at the preantral stage of development. Figure 5C illustrates that the largest follicles in the ovaries of FSH + DHT-treated HYPOXD rats were quite small, possessed a limited complement of granulosa cells, displayed little stimulation of their thecal shells, and displayed no particular "pattern" of appearance in these ovaries. Further, the stromal-interstitial tissue in the ovaries of these animals did not possess a stimulated appearance. In marked contrast, Fig. 5D illustrates that the largest follicles in the ovaries of FSH + DHT + E2-treated HYPOXD rats were at the small antral stage of development, displayed thecal shells with a strikingly stimulated appearance, possessed a remnant of granulosa cells or were in the process of losing granulosa cells, were not involuted, and were arranged in a "string of pearls" motif. In addition, the stromal-interstitial tissue in the ovaries of these animals possessed a stimulated appearance in distinct contrast to the ovarian morphology of FSH + DHT-treated HYPOXD rats (Fig. 5C). Together, these observations provide strong, direct evidence that estrogen plays an important role in the development of the cystic and precystic follicles observed when HYPOXD rats are exposed to tonic stimulation by FSH + A4.

Figure 6 illustrates that placebo- and DHT-treated HYPOXD rats possessed similar mean ovarian LCAs (1.93 ± 0.33 and 1.80 ± 0.34 mm^2, respectively). These values were significantly less than the mean ovarian LCA observed for DHT + E2-treated animals (3.61 ± 0.81 mm^2, $p \leq 0.05$). It is interesting to note that the mean ovarian LCA for FSH + DHT-treated HYPOXD rats (2.64 ± 0.34 mm^2) was similar to that for DHT-treated HYPOXD rats and was significantly smaller than the mean

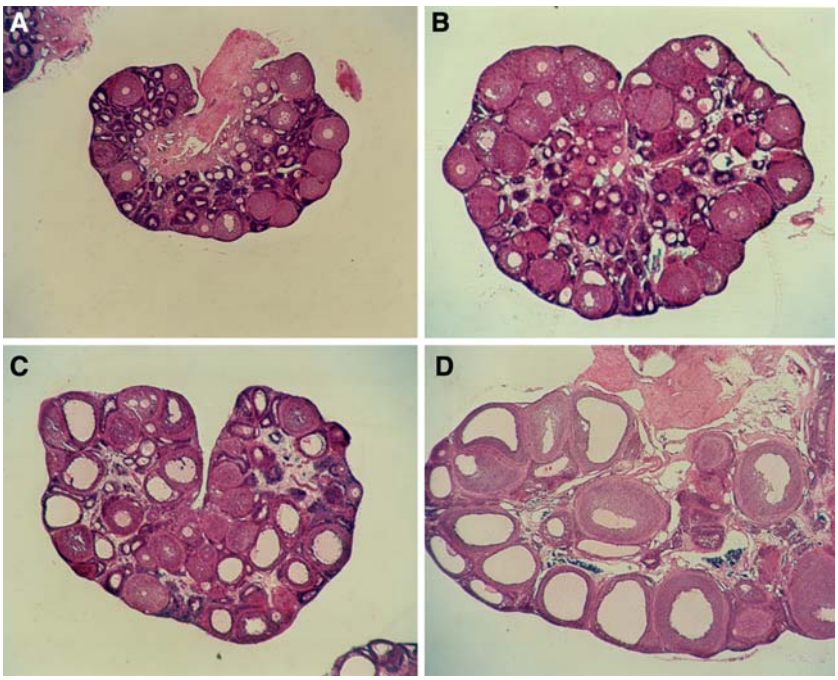

Fig. 5 Impact of DHT either alone or in combination with E2 and or FSH on ovarian morphology of the HYPOXD rat. Ovarian cross-sections from rats bearing 5 mg pellets of DHT (**A**) alone; or with (**B**) a pellet containing 5 mg E2; (**C**) daily treatments for 13 days with 2 μg oFSH; or (**D**) E2 + FSH. Photomicrographs were obtained using a Zeiss IM35 microscope with a 2.5× objective

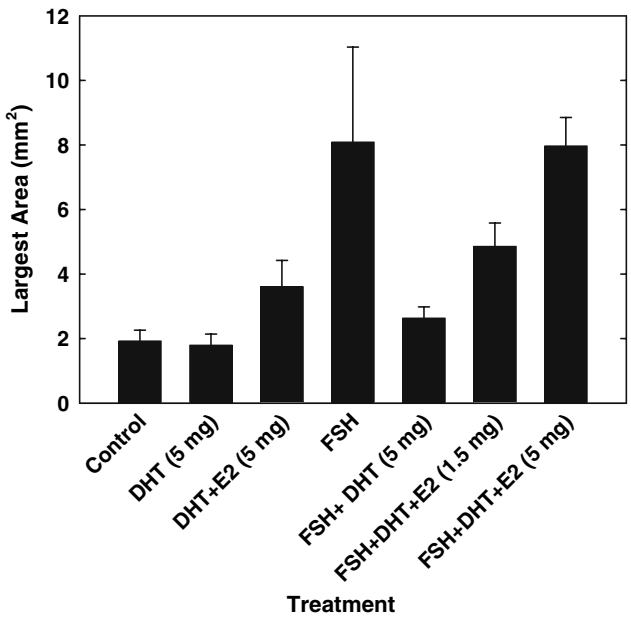

Fig. 6 Impact of DHT, E2, and FSH on the largest cross-sectional areas (LCAs) of ovaries from HYPOXD rats. Each bar represents the mean ± SEM of the average, largest ovarian cross-sectional area for each rats in each treatment group from at least two experiments. Significant differences ($p \leq 0.05$) between groups are described in the text

ovarian LCA for FSH-treated HYPOXD rats (8.09 ± 2.94 mm^2, $p \leq 0.05$). However, inclusion of E2 with the FSH + DHT treatments in vivo resulted in a dose-related reversal of the negative impact of DHT on the ability of FSH to increase the mean ovarian LCA in HYPOXD rats. As shown in Fig. 6, the maximum LCA observed in response to combined treatment with FSH + DHT + E2 was 7.92 ± 0.88 mm^2 which was similar to that observed for FSH + E2-treated HYPOXD rats in Fig. 2 (7.8 ± 1.1 mm^2).

Figure 7 illustrates that, as with A4 treatments alone, the diameters of the largest preantral follicles in the ovaries of DHT-treated HYPOXD rats were similar to those of placebo-treated animals (0.31 ± 0.02 and 0.28 ± 0.01 mm, respectively). Further, the diameters of the largest preantral follicles in the ovaries of DHT + E2-treated HYPOXD rats were larger (0.37 ± 0.01 mm, $p \leq 0.05$) than those of both the placebo-treated and the DHT-treated animals.

The diameters of the largest follicles in the ovaries of FSH + DHT-treated HYPOXD rats (0.437 ± 0.043 mm) were larger than those of the preantral follicles in ovaries of placebo bearing HYPOXD rats ($p \leq 0.05$), were similar in size to the preantral follicles observed in ovaries from DHT + E2-treated animals, yet were smaller than the antral follicles in the ovaries of FSH-treated HYPOXD rats (0.611 ± 0.068 mm, $p \leq 0.05$). Thus, it is difficult to designate the largest follicles in the ovaries of the FSH + DHT-treated HYPOXD rats as either very large atretic preantral follicles or very small atretic antral follicles.

Inclusion of increasing doses of E2 in the treatment of HYPOXD rats with FSH + DHT reversed the negative impact of DHT on the FSH-induced increase in follicular size ($p \leq 0.05$). The maximum ovarian follicular diameter observed in the ovaries of FSH + DHT + E2-treated HYPOXD rats was similar to that observed for FSH-treated HYPOXD rats (0.63 ± 0.02 and 0.61 ± 0.07 mm, respectively).

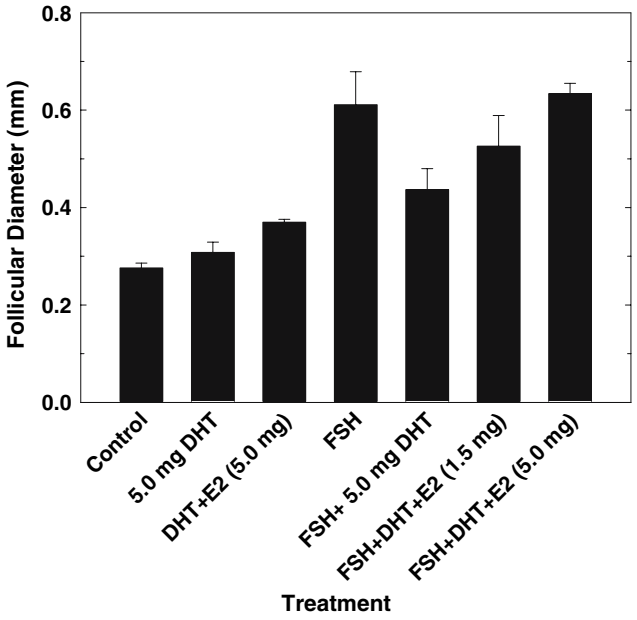

Fig. 7 Impact of prolonged stimulation by DHT, E2, and FSH on the diameters of ovarian follicles in HYPOXD rats. Each bar represents the mean ± SEM of the diameters of the largest follicles present in the ovaries of each rat in the indicated treatment groups. Values were obtained using the ovarian histological sections used to calculate LACs in Fig. 6. Significant differences ($p \leq 0.05$) between groups are described in the text

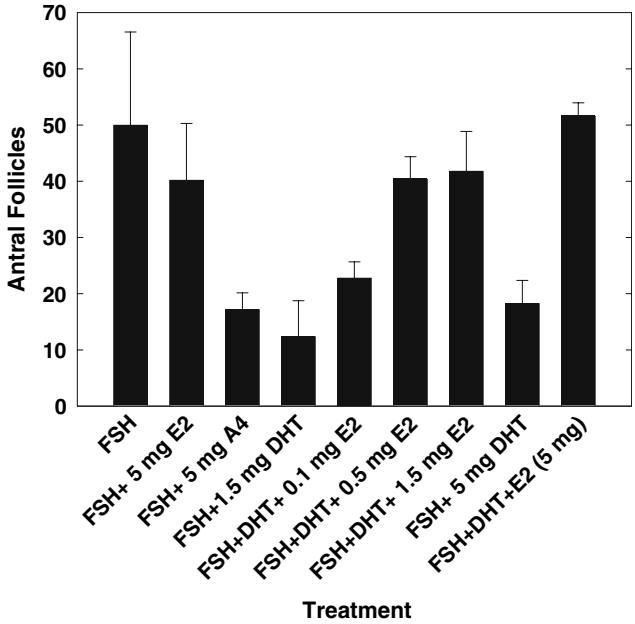

Fig. 8 Impact of prolonged hormonal stimulation on the number of antral or cystic follicles present in the ovaries of HYPOXD rats. Each bar represents the mean ± SEM for the number of antral (healthy or atretic) or cystic follicles present in the ovaries of each rat in the indicated treatment groups. Values were obtained using the ovarian histological sections used to calculate LACs in Figs. 4 and 6. Significant differences ($p \leq 0.05$) between groups are described in the text

Impact of the in vivo hormonal treatments on the number of antral ovarian follicles

Figure 8 illustrates the impact of steroids on the ability of FSH to induce antral follicles in the ovaries of HYPOXD rats. Briefly, the number of antral follicles present in the LCAs for each pair of ovaries from each rat was counted and the mean was calculated for each treatment group. Thus, FSH-treated HYPOXD rats displayed a mean of 50.0 ± 16.6 atretic, small antral follicles per pair of ovarian LCAs by the morning of day 14. FSH + E2-treated HYPOXD rats displayed a similar number of small antral follicles per pair of ovarian LCAs per rat (40.2 ± 10.1 small antral follicles). In marked contrast, HYPOXD rats treated with FSH plus 5 mg pellets of either A4 or DHT displayed significantly fewer small antral follicles (17.0 ± 3.0, 18.3 ± 4.1, respectively) than FSH ± E2-treated HYPOXD rats ($p \leq 0.05$).

As illustrated in Fig. 8, inclusion of pellets with increasing doses of E2 (0.1–1.5 mg pellets) in the treatment regimen of FSH-treated HYPOXD rats bearing a 1.5 mg DHT pellet resulted in a dose related increase in the number of antral, cystic ovarian follicles observed in the LCAs of these treatment groups ($p \leq 0.05$). A similar maximal increase in the number of cystic follicles was observed when a 5 mg E2 pellet was included in the treatment regimen of FSH-treated HYPOXD rats bearing a 5 mg DHT pellet. The maximum numbers of cystic follicles observed in the LCAs of FSH-treated HYPOXD rats bearing a 1.5 or 5 mg DHT pellet plus an E2 pellet of similar dosage were similar to the number of atretic antral follicles observed in the ovaries of FSH-treated HYPOXD rats (41.8 + 7.1, 51.6 + 2.4, and 50.0 + 16.6 follicles, respectively).

Discussion

The present study is an important extension of our previous work, which demonstrated that prolonged stimulation by FSH plays not only a permissive role [26] but also an obligatory role [29] in the induction of *large* polyfollicular ovarian cysts in hCG-treated HYPOXD rats. In contrast to the preceding observations, the present results clearly demonstrate that prolonged exposure of FSH-treated HYPOXD rats to A4 or E2 specifically results in the induction of *small* ovarian cysts, approximately 0.61–0.65 mm in diameter in contrast to the 3–5 mm follicular cysts observed in the ovaries of FSH + hCG-treated HYPOXD rats [26, 29]. In addition, the present results show that

prolonged tonic exposure to DHT, a strong non-aromatizable androgen, cannot induce either large or small follicular cysts in the ovaries of FSH-treated HYPOXD rats. In fact, tonic exposure of FSH-treated HYPOXD rats to either A4 or DHT results in a 60% decrease in the number of follicles recruited to the antral stage of development compared to the number of antral follicles recruited in the ovaries of HYPOXD rats treated with FSH alone. In addition, the diameters of the largest follicles in the ovaries of FSH + DHT-treated HYPOXD rats are markedly smaller than those observed for FSH + A4- or FSH + E2-treated HYPOXD rats. Together, these results provide strong evidence that "pure" androgen action is not a key factor in the induction of the *small* follicular cysts observed in the ovaries of FSH + A4-treated HYPOXD rats.

The present results provide strong evidence to support the idea that estrogen-like action plays an obligatory role in the induction of the *limited* number of *small* follicular cysts observed in the ovaries of FSH + A4-treated HYPOXD rats. These observations, and the ability of increasing doses of E2 to reverse the suppression, by DHT, of the recruitment of preantral ovarian follicles to the antral stage by FSH, also argue strongly for an obligatory role for prolonged exposure to estrogen in the development of *polyfollicular* small ovarian cysts in FSH + E2 ± DHT-treated HYPOXD rats.

The spontaneous expression of ovarian cysts is a common occurrence in many mammalian species [1–17], and is the leading cause of infertility in women [15–17, 31]. The heterogeneous endocrine environments associated with an established cystic ovary state suggests that cystic ovaries develop in response to one or more disruptions of normal interactions within the complex polygenetic relationships that regulate the metabolic environment and the entrainment of reproductive cyclicity [15, 31]. Without reproductive cyclicity, the ovaries are exposed to tonic stimulation by pituitary factors as well as factors produced by the ovary and/or peripheral tissues that result in the specific development of large or small ovarian cysts and the establishment of a cystic ovary state. Due to logistical and ethical considerations, however, little direct evidence is available regarding the specific factors that actually are involved in driving the *spontaneous* development of polyfollicular ovarian cysts in higher order species such as cows and women.

For example, endocrinopathies such as hyperandrogenism (HA) and hyperinsulinemia-insulin resistance (HIR) are observed in many, but not all, women diagnosed with polycystic ovary syndrome (PCOS [15–17, 31]). Thus, it has become generally accepted that HA and HIR play important roles in the etiology or maintenance of the "PCO" state in these women. However, the purported roles for HA or HIR in the etiology of such a state have yet to be tested in an unambiguous manner. Thus, it is not clear at this time if the ostensible roles for HA and HIR in PCOS lie primarily at the level of disruption of neuroendocrine-ovarian feedback mechanisms, at the level of the ovary, or at both sites.

Similarly, although elevated peripheral concentrations of estrogens are consistent characteristics of established cystic ovary states in mammals, little has been done to evaluate, unambiguously, the potential role for estrogen at the level of the ovary in the development of ovarian follicular cysts. Previous attempts to study and understand the mechanisms involved in the induction of cystic ovary states have used primarily the rat and, to a much lesser degree, other rodents and mammals [1–14, 18–26, 32–38]. Our previous work [26, 29], as well as the results of the studies presented here, indicate that the HYPOXD rat can be used to examine the potential for specific gonadotropin and steroid hormones to drive the induction of follicular cysts at the level of the ovary in vivo in much the same manner as the HYPOXD rat has been used to elucidate the roles of specific hormones in normal follicular development [39, 40].

A4 was chosen as one of the treatment hormones in the first series of experiments because serum values for both A4 and E2 rise and fall in parallel with the development of large ovarian cysts in FSH + hCG-treated HYPOXD rats [26]. By the end of the in vivo treatments, HYPOXD rats bearing A4-pellets displayed elevated testosterone, A4, E2, and E1 compared to HYPOXD rats bearing placebo pellets. Since the rat ovary possesses a very active 17-ketosteroid reductase that does not appear to be regulated by gonadotropins at any stage of follicular development [30], it is not surprising to see significant yet low amounts of testosterone, E2, and even E1 in the sera of these animals on day 14 of the in vivo treatments. Still, it is not certain if these concentrations of steroid were due solely to metabolism of A4 in the HYPOXD rats or if the pellets were directly responsible for some of the testosterone, E2, and E1 observed in the sera of these animals.

The marked difference in the ovarian morphology observed for FSH + A4-, FSH + E2-, and FSH + DHT-treated HYPOXD rats suggests that the amount of cross-over steroids observed in the sera of each treatment group had no major impact on the interpretation of the results. For example, the highly stimulated appearance of the thecal shells in the animals bearing E2 implants (in the presence or absence of FSH) appears to be an estrogen effect since thecal tissue from HYPOXD rats bearing DHT implants (in the presence or absence of FSH) fail to develop a similar stimulated appearance. It is well established that E2 stimulates the production of a number of factors by granulosa cells that have a regulatory impact on thecal cell development. The present results suggest that unabated

exposure of thecal and stromal tissue to these FSH + E2 responsive factors may permit the development of small polyfollicular cysts rather than atretic follicles in FSH + E2 ± DHT-treated HYPOXD rats.

Although combined treatments with gonadotropins and steroids had no sustained, impact on serum steroid concentrations in these animals by the morning of day 14, this does not mean that these combined treatments had no impact on serum steroid values early in the in vivo treatment regimens. Our previous observations clearly demonstrate that serum A4 and E2 values specifically rise and fall during the induction of large polyfollicular cysts in response to prolonged, combined stimulation by FSH + hCG in vivo [26]. Thus, it is possible that, during the initial induction of the limited number of cysts and precysts observed in the FSH + A4-treated HYPOXD rats, a similar pattern of a rise and fall in ovarian produced estrogen values may occur as the follicles commit to developing into small ovarian cysts. Further research will be required to determine if such a pattern of E2 production by the ovary is an important aspect of determining whether a limited number or large cystic follicles or a large number of small cystic follicles will develop in response to specific hormonal stimuli.

Ovarian-produced steroids display concentrations in mammalian follicular fluid that are routinely 20- to 1,000-fold greater than their concentrations in peripheral serum [41–46]. The amount of steroid observed in the peripheral serum of the HYPOXD rats bearing steroid pellets appears to be within the physiologic range for rats. For example, fluid from preovulatory rat follicles characteristically displays E2 concentrations of ~39 ng/ml [41]. Although the concentration of E2 in follicular fluid from small and medium antral rat follicles is not known, the amount of E2 in the sera of HYPOXD rats treated with E2 pellets is at least 6-fold less than the values observed in preovulatory rat follicles [41].

Similarly, although values do not appear to be available for A4 concentrations in rat follicular fluid, concentrations of A4 in the follicular fluid of small to medium sized follicles from other mammalian ovaries can be 20–500 ng/ml and increase further in preovulatory follicles [42–45]. Therefore, as with E2, the concentrations of A4 in the sera of HYPOXD rats bearing A4 pellets appears to be within the expected physiologic range for small and medium sized follicles. Finally, Tetsuka and Nancarrow have demonstrated that similar amounts of DHT are present in follicular fluid from healthy and atretic ovine follicles [46]. This observation led them to suggest that DHT does not have a negative role in determining the fate of follicles in sheep (which tend to be monovulatory). Our previous observations, that small antral follicles from control rats and preovulatory follicles from hCG-treated immature rats display the same *capacity* to produce testosterone, DHT, 5α-androstane-3α,17β-diol, and androsterone in vitro (30), indirectly support this idea. Thus, although the impact of DHT on ovarian cyst development in HYPOXD rats remains to be elucidated, it appears from the data obtained in this series of experiments that DHT does not play a direct, obligatory role at the level of the ovary in the induction of polyfollicular cysts in the HYPOXD rat.

The mechanisms by which FSH, E2, and DHT act together to induce the development of small polyfollicular cysts in a "string of pearls"-like motif in the ovaries of HYPOXD rats remain to be elucidated. Still, the striking difference in the appearance of FSH + hCG-induced large polyfollicular ovarian cysts and the FSH + E2 ± DHT induced small polyfollicular ovarian cysts in HYPOXD rats indicate that these models will provide exciting new insight into the specific mechanisms by which specific hormonal environments induce specific types of ovarian cysts.

As with any animal model for normal ovarian follicular development, care must be taken when attempting to extend empirical observations in one species to hypotheses regarding a similar situation in other species. The only way to ascertain the true relevance of this model to humans and other monovulatory mammals will be to test directly the impact of these hormones on ovarian cyst development in these higher order species. Thus, the growing body of evidence in several species (18–25) that tonic stimulation by subovulatory amounts of LH-like activity can induce a physiologic number of large ovarian cysts, raises important questions about generally held, yet still untested, concepts regarding the hormones and mechanisms involved in the etiology of small polyfollicular ovarian cysts in mammalian ovaries.

The established human polycystic ovary has long been described as possessing a highly stimulated appearance [16], albeit these comments have been directed at the thecal and stromal-interstitial tissue of these ovaries until recently. Our previous observation that the induction of large ovarian cysts by subovulatory doses of hCG in *both* neuroendocrinologically suppressed rats [18, 19] and FSH-treated HYPOXD rats [26] does not require the inhibition of granulosa cell aromatase activity has been expanded, by others, to human granulosa cells from small ovarian follicles from women with PCOS [47]. Together, these observations were the first to suggest a potential role for estrogens at the level of the mammalian ovary during the development of cystic follicles. Subsequent research has demonstrated that human granulosa cells from some women with PCOS are hypersensitive to FSH [31] in contrast to the generally held notion that these granulosa cells should be insensitive to FSH as a result of tonic exposure to acyclic amounts of LH and androgens. Together, these observations with rat and human tissues indicate at least

some potential agreement between the mechanisms underlying the development of the "string of pearls" ovary in the FSH + E2 + DHT-treated HYPOXD rat and the development of the "string of pearls" ovarian morphology in at least some women with PCOS [16, 47]. Further research with ovarian tissue from rats and other higher order mammals will be required to determine if similar hormones and subcellular mechanisms are involved in the general induction of specific types of mammalian ovarian cysts.

The mechanisms involved in follicular selection and follicular development appear to remain intact in neuroendocrinologically suppressed intact rats during the induction of ovarian cysts with subovulatory doses of hCG [18–20] but not in HYPOXD rats treated with FSH alone [26, 29], or FSH + hCG [26, 29], or FSH + E2 ± DHT. The observations that FSH + hCG-treated HYPOXD rats develop large *polyfollicular* cysts and FSH + E2 ± DHT-treated HYPOXD rats develop small *polyfollicular* cysts, whereas FSH + A4- and FSH + DHT-treated HYPOXD rats develop only a limited number of cysts or antral follicles, respectively, indicate that a neuroendocrine factor other than LH modulates the ability of FSH to recruit preantral follicles to the antral stage of development. Although the identity of this putative factor remains to be determined, the ability of A4 to perform this task supports the idea that this neuroendocrine factor may act, in part, by promoting ovarian production of A4 and its metabolism to strong androgens such as testosterone and DHT at specific stages of follicular development.

The trophic effects of LH play a major role in determining the optimal size of developing preovulatory follicles as well as their maximal ability to produce E2 [39, 40, 48, 49], in addition to its ability to stimulate ovarian thecal and stromal function. Yet in the present series of experiments, the ovarian thecal and stromal tissues display a highly stimulated appearance in the absence of LH. Further, the largest ovarian follicles in HYPOXD rats treated with FSH + E2 ± DHT in this series of experiments are at the small antral stage of development. This is in marked contrast to the large polyfollicular cysts observed in FSH + hCG-treated HYPOXD rats [26, 29]. These observations raise important questions regarding the actual hormonal interactions that result in hyperstimulated ovaries with small polyfollicular cysts in other mammals [1–18]. To our knowledge, the data presented in this series of experiments is the first to directly address the conceptual dichotomy between the enlarged, generally stimulated appearance of ovaries bearing *small* polycystic follicles and the obligatory role of LH in determining the final optimal size for normally developing preovulatory follicles as well as the ability of unabated stimulation by subovulatory doses of LH-like activity to drive the specific development of large ovarian cysts in mammalian ovaries.

DHT can inhibit the expression of LH receptors on the granulosa cells of immature rats in response to FSH [48], whereas prior treatment of similar animals with E2 can block the effect of DHT [48]. This observation and the data presented here regarding the roles for FSH and E2 in the induction of small polyfollicular cysts in HYPOXD rat ovaries support the concept that the expression of LH receptors on granulosa cells at a much earlier stage of follicular development (3 mm) in a cohort of women with PCOS than observed in normally cycling human ovaries (10 mm) [31] may be due to tonic exposure of the ovaries to the slightly elevated estrogenic environment known to be associated with the established PCOS state. Further work using the FSH + E2 ± DHT-treated HYPOXD rat will provide a mechanistic explanation for the development of small polyfollicular cysts in mammalian ovaries that can be tested using ovarian tissue from other mammalian species.

The development of normal preovulatory follicles requires precise timing among the interactions of FSH, LH, and E2 at the level of the ovary. It is well-established that androgen receptors are present in ovarian tissues [39, 40] and that androgens have a stage specific positive or a negative impact on follicular development that goes beyond the role of aromatizable androgens acting as substrates for the production of E2 [49–54]. It is also well documented that estrogen receptors (ERs) and/or mRNA for these receptors are present in the ovaries of several mammalian species including rats and humans [55–71]. The content of the ERα and ERβ forms of the ER in the ovary appears to be specific to the stage of development of the follicles as well as to the ovarian tissue itself in several mammalian species [55–71]. Indeed ERβ appears to play an obligatory role in the early- and mid-stages of normal follicular development whereas ERα does not. Further, mice, in which only ERβ is expressed (αERKO mice) develop large polyfollicular cysts similar to those observed in the FSH + hCG-treated HYPOXD rat [26, 29, 68, 69], which suggests a role for the ERβ in at least the development of this type of ovarian cyst.

Although we do not have data regarding the impact of the hormonal treatments in this present series of experiments on ovarian ER content, there is evidence to suggest that DHT may modulate the number of follicles that FSH can recruit to the antral stage of development by modulating the presence of ERα in preantral follicles during the early follicular phase of the cycle when estrogen values may be too low to oppose the effect of DHT [46, 70]. As estrogen increases during normal follicular development, it takes on the role of an antiandrogen [72]. Therefore, it is possible that in the FSH + E2 ± DHT-treated HYPOXD rat, in which small follicles are exposed to quantities of estrogen that are similar to those experienced by small and medium-sized growing follicles, there may be a disruption

of the normal timing of both thecal and granulosa cell gene expression. Whether or not this paradigm may lead to the precocious expression of LH receptors on granulosa cells or the inappropriate expression of other factors that impact on thecal growth and development [73–76] and the development of ovarian cysts in the present model remains to be determined.

In summary, the present series of experiments clearly shows that (1) unabated tonic stimulation by highly purified FSH and estrogen can induce small polyfollicular cysts in the ovaries of immature HYPOXD rats; (2) unabated stimulation by the non-aromatizable androgen, DHT, cannot replace A4 in the induction of small ovarian cysts observed in FSH + A4-treated HYPOXD rats; and (3) combined stimulation by FSH + E2 + DHT results in the induction of ovarian cysts in a "string of pearls" motif in HYPOXD rats that is reminiscent of the classic gross ovarian morphology associated PCOS in women [16]. Thus, prolonged exposure to increasing amounts of E2 not only appears to override the negative impact of androgens on the ability of FSH to recruit large numbers of preantral follicles to the small antral stage of development in FSH + DHT-treated HYPOXD rats but also appears to promote the development of *polyfollicular* cysts directly at the level of the ovary in these animals. These observations provide exciting new insights and raise tantalizing questions regarding the hormonally driven mechanisms involved in the switching on and off of genes that are involved in the induction of large or small ovarian cysts in other mammalian species, including humans.

Materials and methods

Animals and ovarian histology

The USC Institutional Animal Care and Use Committee approved the in vivo research protocol. Briefly, immature female rats, hypophysectomized at 21 days of age, were obtained from Charles River (Boston, MA). To help maintain viability these animals were fed oranges in addition to the rat chow provided by the USC Animal Resources Facility, and were provided with water containing 10% sucrose. The animals were housed in plastic containers in a temperature-controlled room with a light:-dark cycle of 12:12.

Because of the prolonged experimental protocol, incompletely HYPOXD rats were easily detected by their ability to sustain a pattern of growth and internal organ development (including corpora lutea in the ovaries of some of these animals), as well as increased trunk blood volumes not observed in completely HYPOXD animals and indicative of the presence of significant amounts of growth hormone in these animals. These growth patterns directly correlated with the presence of pituitary remnants in these animals. Tissue from animals that were incompletely HYPOXD was discarded at the end of the in vivo treatment regimens (up to 40% of each and every set of animals purchased). Replacement HYPOXD rats were obtained and experiments were repeated until tissue was available from a statistically significant number of completely HYPOXD rats in each treatment group.

Our initial goal was to obtain an "*n*" of 6 for each dose of steroid used alone and in combined treatments with gonadotropins and/or other steroids. However, because of the difficulty in obtaining completely HYPOXD rats, and because the largest follicles in all treatments groups that did not receive FSH were at the preantral stage of development, and since virtually identical data were obtained within specific *types* of hormonal treatments of completely HYPOXD rats that received either (1) increasing doses of specific individual steroids; or (2) increasing doses of specific individual steroids plus hCG; or (3) increasing doses of E2 + A4, or (4) increasing doses of E2 + DHT … we stopped obtaining animals for the groups that only produced preantral follicles as soon as we were able to obtain at least three completely HYPOXD rats for each tested dose of hormone. Thus, $n = 3$–4 for completely HYPOXD animals bearing only 1.5 mg or 5 mg cholesterol-, A4-, or E2-pellets, $n = 3$ or 6 for completely HYPOXD rats treated with 1.5 mg or 5 mg DHT pellets; $n = 3$ or 4 for completely HYPOXD rats bearing increasing doses of E2 *plus either* A4- *or* DHT-pellets; and $n = 3$ for all doses of A4 or E2 used in hCG-treated HYPOXD rats. During the course of these same experiments we were able to obtain the following numbers of completely HYPOXD rats for in vivo treatments with FSH plus increasing doses of cholesterol or steroid containing pellets: FSH plus placebo, $n = 6$; A4 + FSH, $n = 5$; E2 + FSH, $n = 5$ or 7; DHT + FSH, $n = 5$ or 7; FSH + E2 + DHT, $n = 4$ for groups bearing 0.1 mg E2 pellets and $n = 5$ or 6 for all other doses of E2 pellets used in the FSH + E2 + DHT treated HYPOXD rats.

This is not the first time that we have observed this high ratio of incompletely HYPOXD animals from various vendors. This observation is an important caveat that completeness of hypophysectomy must be ascertained whenever research protocols depend on obtaining tissue from short-term hormonally stimulated HYPOXD rats.

In the first series of experiments, HYPOXD rats were given sc either (a) a placebo pellet containing 1.5 mg or 5.0 mg cholesterol (controls); (b) 1.5 mg or 5.0 mg A4 pellets (21-day release, Innovative Research of America [IRA], Sarasota, FL); (c) 1.5 mg or 5.0 mg E2 pellets (21-day release, IRA); (d) A4 plus E2 pellets; (e) A4 pellets plus twice-daily injections of 1 IU hCG (4,000 IU/mg,

Sigma Chemical Co, St. Louis, MO) in phosphate buffered saline, pH 7.0, containing 0.09% pig skin gelatin (gel-PBS) for 13 days beginning on the second day after the steroid implants were administered; (f) A4 pellets plus daily injections of 2 μg FSH (10 U/mg; NIADDK-oFSH-17; <0.04 times NIH-LH-S1) in gel-PBS; (g) E2 pellets plus injections of hCG; or (h) E2 pellets plus injections of FSH.

In the second series of experiments, HYPOXD rats were given either (a) a placebo pellet; (b) 1.5 mg or 5.0 mg DHT pellets (21-day release, IRA); (c) DHT pellets plus pellets with similar doses of E2; (d) DHT pellets plus daily injections of FSH starting on the second day after the implants were inserted sc; or (e) DHT + FSH plus pellets with increasing doses of E2 (0.1, 0.5, 1.5, 5.0 mg; 21-day release, IRA).

Between 0730–0830 h on day 14 of gonadotropin treatment, animals were anesthetized with pentobarbital, decapitated, trunk blood was collected in polypropylene tubes and placed on ice, and ovaries were excised. Ovaries from each animal were fixed in formalin and subsequently sectioned and stained with eosin and hematoxylin by the USC School of Medicine Histology Core Facility. Photomicrographs were obtained at 2.5× with a Zeiss 35IM microscope. Histological sections were obtained and the sections with the largest ovarian cross-sections were used to observe ovarian and follicular morphology.

The largest ovarian cross-sectional area per ovary for each rat was calculated using the formula "$\pi \times r_1 \times r_2$" where r_1 equals one-half of the longest length and r_2 equals one-half of the longest width for each ovary. The average of these largest ovarian cross-sectional areas was calculated for each pair of ovaries from each rat, and the mean ± SEM of these averages was calculated for each treatment group. These dimensions were obtained with an ocular micrometer. Ovarian follicular diameters also were obtained with an ocular micrometer; however, the ovarian sections that displayed the largest follicular diameter were used to obtain these values rather than simply using the histological section with the largest ovarian cross-sections.

Preparation of serum samples for radioimmunoassay of steroid content

Serum samples were harvested and stored in individual polypropylene tubes at –20°C according to protocols routinely used in this laboratory [26]. Save for the antisera against DHT, which has a cross reactivity of ~10% with testosterone, the antisera used to assess serum steroid content are highly specific for the steroids against which they were generated. Nevertheless, to ensure that there would be no interference in the analysis of the steroids of interest, and in order to analyze each steroid of interest in the very small individual volumes of serum obtained from completely HYPOXD rats, each serum sample was extracted and individually subjected to high-pressure liquid chromatography (HPLC) chromatography [26] prior to analysis by RIA.

Briefly, a known amount (1,500 cpm) of the tritiated form of each steroid of interest (PerkinElmer Life Sciences, Boston, MA) was added to a known volume of each serum sample. The samples were vortexed, warmed at 45°C for 30 min, brought to room temperature, and extracted using filtered HPLC grade diethyl ether (Fisher Scientific Co, Pittsburgh, PA). The ether extract from each sample was dried in its individual conical tube in a Savant centrifugal evaporator. The resulting residues were dissolved in filtered, HPLC grade acetonitrile (AcN, Fisher Scientific Co) with vortexing and warming. Each sample was brought to a final AcN:H_2O ratio of 40:60 with filtered MilliQ grade water and HPLC grade AcN just prior to injection onto a 25 cm Rainin C-18 microsorb, end-capped column. Chromatography was performed at a flow rate of 1.5 ml/min using a 30 min isocratic AcN:H_2O (37:67) gradient followed by a 1 min linear gradient to 100% AcN. Column effluent was collected as 0.4 min samples using a Gilson FC-80 fraction collector. Fractions associated with the steroids of interest were harvested, dried, reconstituted with 1 ml gel PBS, warmed at 45°C for 30 min with vortexing, and stored at –20°C until analyzed by radioimmunoassay (RIA) for steroid content.

RIAs

Antisera against E2 was the generous gift of Dr. Gordon Niswender (Fort Collins, CO). Antisera against A4 and testosterone were generously provided by Drs Gerry Nordblom and Barry England (Ann Arbor, MI). Antisera against DHT was obtained from Dr. PN Rao (San Antonio, TX). Antisera against E1 and its iodinated ligand were obtained from Pantex (Santa Monica, CA). The iodinated forms of E2, testosterone, and A4 were obtained from Diagnostic Systems Laboratories (Webster, TX). Tritiated DHT was obtained from DuPont New England Nuclear (Boston, MA) and chromatographed before use. RIAs were performed as previously described [18, 19, 26, 29, 30]. Intra- and interassay coefficients of variation were: A4 (6% and 18%), DHT (8% and 17%), E2 (6% and 10%), E1 (7% and 12%), and testosterone (4% and 10%).

Statistics

Statistical differences ($p \leq 0.05$) between and among treatment groups were determined using the SigmaStat Statistical Program's (Jandel Scientific, San Rafael, CA) ANOVA and Student–Neuman Keuls after-test to isolate differences ($p \leq 0.05$).

Acknowledgements The author wishes to thank Mr. Thomas Sanford and Mr. Thomas "Hoke" Anderson-Currie for their technical assistance with the in vivo treatments as well as the preparation and analysis of the serum samples for steroid content.

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