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Effect of azaline B on follicular development and functions in the hamster

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Abstract

The usefulness of azaline B, a GnRH antagonist, in suppressing gonadotropin secretion in the golden hamster was examined by examining follicular development, steroidogenesis and expression of steroidogenic enzymes. Serum levels of P and E declined significantly, while FSH or LH was undetectable in azaline B-treated hamsters. FSH significantly increased serum E levels, whereas LH upregulated serum P levels. The formation of antral follicles ceased in azaline-treated hamsters, but was reversed by FSH with or without LH supplement. FSH also activated the primordial follicle pool resulting in increased formation of primary and preantral follicles. Further, an increasing trend in the formation of preantral follicles in response to E or E + P, and the formation of antral follicles in response to E + P treatment was evident. The level of Cyp11a1 mRNA increased markedly in LH- or LH + FSH-treated hamsters, whereas FSH with or without LH upregulated Cyp17a1, Cyp19a1 and Fshr mRNA expression. E without or with P also upregulated ovarian Cyp19a1 mRNA expression. The expression of enzyme protein corroborated the mRNA data. In summary, azaline B is an efficient GnRH antagonist in the hamster, and will be useful in studying the selective effect of gonadotropins on ovarian functions without disrupting the physiological functions of other hormones in ovarian cells.

Keywords

Azaline B; FSH; LH; Ovary; Follicles

1. Introduction

Gonadotropins are the most important regulators of ovarian functions in mammals (Greenwald and Roy, 1994). Gonadotropins are secreted from the anterior pituitary upon pulsatile stimulation of hypothalamic gonadotropin-releasing hormone (GnRH). Follicle stimulating hormone (FSH) and luteinizing hormone (LH) regulate ovarian steroidogenesis (Matsuda et al., 2012). FSH acts on the granulosa cells to control follicular growth and

positively regulates estradiol-17 β (E) production. LH acts on theca cells to produce androgens, which are used as a precursor for E synthesis in granulosa cells (Gore-Langton and Armstrong, 1994). Gonadotropins regulate steroid production in the ovary by activating various signaling pathways that leads to the expression of steroidogenic enzymes (Richards et al., 2002a, 2002b). The unique expression pattern of steroidogenic enzymes in follicular cells determines reproductive cycle-specific ovarian steroid production. Important enzymes regulating ovarian steroidogenesis are cholesterol side-chain cleavage (CYP11a1), 17 α -hydroxylase/17,20 lyase (CYP17a1) and aromatase (CYP19a1) (Gore-Langton and Armstrong, 1994; Richards, 1994). CYP11a1 catalyzes the conversion of cholesterol to pregnenolone, while CYP17a1 catalyzes two reactions, hydroxylation of pregnenolone or progesterone at C17 and conversion of C17 steroid substrate into C19 products (Gore-Langton and Armstrong, 1994). CYP19a1 converts androstenedione or testosterone to yield estradiol-17 β (E) and estrone (Gore-Langton and Armstrong, 1994; Pan et al., 2012; Patel et al., 2010; Payne and Hales, 2004; Rani and Moudgal, 1978). E acts on follicular cells primarily through estrogen receptor α (ESR1) and estrogen receptor β (ESR2) although recent evidence suggests that transmembrane estrogen receptor may also mediate the non-genomic action of estrogen in the ovary (Chakraborty and Roy, 2013; Wang et al., 2007). E upregulates gonadotropin receptor expression in rat granulosa cells and baboon fetal ovaries (Richards et al., 1976a; Zachos et al., 2003), and increases ESR1 and ESR2 expression in rat granulosa cells (Britt et al., 2004; Drummond et al., 1999; Richards, 1975) or in the ovary of hypophysectomized hamsters (Yang et al., 2002). E also plays an important role in ovarian follicle development (Britt et al., 2004; Sugiura et al., 2010).

GnRH regulates pulsatile gonadotropin secretion from the gonadotropes (Krsmanovic et al., 2010) and acts via GnRH receptors (McArdle et al., 2002). GnRH receptors can be downregulated by GnRH agonists leading to inhibition of gonadotropin secretion, and consequent suppression of ovarian functions (Fraser, 1993). However, GnRH agonists *in vivo* or *in vitro* have been shown to directly inhibit ovarian steroidogenesis in the rat as well as in the human (Otani et al., 1982; Uemura et al., 1994), suppresses LH-receptor expression (Jones and Hsueh, 1980) and directly suppresses luteal progesterone synthesis in rats (Clayton et al., 1979). Another side effect of GnRH agonists is the initial surge of gonadotropin secretion, which compromises their clinical application. On the other hand, GnRH antagonists strongly compete for the GnRH receptors and suppress GnRH-induced GnRH receptor gene expression resulting in immediate, dose-dependent suppression of gonadotropin secretion without an initial stimulation of the gonadal axis (Gobello, 2012). GnRH agonists and antagonists have been used in the treatment for various clinical conditions such as breast cancer, prostate cancer, endometriosis, premenstrual syndrome, infertility (Karten and Rivier, 1986; Tarlatzis and Bili, 2003), precocious puberty (Mul and Hughes, 2008; Schultze-Mosgau et al., 2005). Azaline B is a synthetic GnRH receptor antagonist that has very low anaphylactic properties (Campen et al., 1995; Rivier et al., 1995a). The effectiveness of GnRH antagonists *in vivo* in the context of gonadotropin-induced folliculogenesis or ovarian steroidogenic enzyme expression in laboratory rodents, particularly in the golden hamster, warrants further study. Conventional hypophysectomy removes other important hormones such as the thyroid, adrenocortical and growth hormones from the system. Deficiencies of these hormones not only affect the physiology of gonadal

cells (Jiang et al., 2000; Maruo et al., 1992; Sasson and Amsterdam, 2003a), but also alter major hormonal regulation of body functions. The objectives of the present study were to characterize the effect of azaline B on folliculogenesis, expression of steroidogenic enzymes, ovarian steroid production and FSH receptor expression in order to determine if azaline B would be suitable to study the specific *in vivo* effects of gonadotropins on ovarian functions. Golden hamsters were selected based on the precise nature of their estrous cycles, well-defined stages of follicular development and serum levels of reproductive hormones corresponding to the estrous cycles (Saidapur and Greenwald, 1978a).

2. Materials and methods

2.1. Antibodies and chemicals

Azaline B was a generous gift from Dr. Jean Rivier (The Salk Institute, San Diego, CA). Antibodies for CYP11a1, CYP17a1, CYP19a1 immunodetection and for progesterone (P) radioimmunoassay (RIA) were kindly provided by Dr. D. C. Johnson and Dr. Michael Soares (University of Kansas School of Medicine, Kansas City, KS); antibody for E RIA was a generous gift from Dr. K. Quadri (Kansas State University, Manhattan, KS), DyLight-conjugated second antibodies were from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA), plastic embedding medium was obtained from Electron Microscopy Sciences (Hatfield, PA), ovine-FSH-20 and ovine-LH-25, and FSH-RIA and LH-RIA kits were provided by Dr. A. Parlow, National Pituitary Hormone Program (Harbor UCLA Medical Center, CA), E and P were from Pharmacia-Upjohn Co. (Kalamazoo, MI), and Steraloids (Wilton, NH), respectively, quantitative RT-PCR primers and probes were synthesized in the Eppely DNA Synthesis Core Facility (University of Nebraska Medical Center, Omaha, NE), RNeasy mini kit and Taq DNA polymerase were from Qiagen, Inc. (Valencia, CA). All other molecular biology grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), United States Biochemical (Cleveland, OH) or Fisher Scientific Corp. (Pittsburgh, PA).

2.2. Experimental design

Female golden hamsters (90–100 g) were obtained from Harlan Sprague Dawley Laboratories and housed in climate-controlled environment with 14 h light and 10 h dark cycle, and fed *ad libitum* according to the UNMC Institutional Animal Care and Use Committee (IACUC) and United States Department of Agriculture guidelines. The use of hamsters in the research was approved by the UNMC IACAC. Animals with three consecutive estrous cycles were used in the experiments. Cyclic hamsters were administered azaline B sc (50 µg/100g body weight) every 48 h from day 1:0900 h. Disappearance of vaginal discharge on the following estrous cycles confirmed the block in gonadotropin secretion. Azaline B-treated hamsters were administered 0.5% BSA in saline sc (vehicle controls), 10 µg ovine-FSH-20 (NIDDK-NIH) at 0900 h and 1600 h on days 11th and 12th, 5 µg ovine-LH-25 (NIDDK-NIH) once at 1400 h on 12th day, or a combination of FSH and LH when LH was injected at 1400 h on 12th day to mimic the preovulatory LH surge. Ovaries were collected at 0900 h on 13th day. In a parallel experiment, azaline B-treated animals were administered a single dose of sesame oil (vehicle control) sc, 100 µg estradiol-17β-cypionate (E), 500 µg progesterone (P), or a combination of E and P on 12th

day at 0900 h. Ovaries were collected 24 h after the treatment, and trunk blood was collected to measure serum levels of FSH, LH, E or P by radioimmunoassay. Ovaries were either fixed in Bouin's fixative for plastic sections, embedded in OCT (optimal cutting temperature) for frozen sections, or flash-frozen in liquid N₂ for total RNA extraction.

2.3. Immunofluorescence localization of steroidogenic enzymes

Frozen ovary sections (6 µm thick) were fixed in freshly prepared 4% paraformaldehyde in PBS (pH-7.4) at 4 °C, and incubated with enzyme-specific antibodies. The signal was developed using donkey anti-rabbit-IgG-DyLight-488 and nuclei were stained with 4',6-diamino-2-phenylindole (DAPI). The images were captured by a Leica DMR microscope (North Central Instruments, Plymouth, MN) and Openlab image analysis software (Improvision, Lexington, MA).

2.4. Expression of Cyp11a1, Cyp17a1, Cyp19a1, β-actin, and Fsh-receptor mRNA

Ovarian total RNA was used for qPCR determination of the levels of respective mRNAs using fluorescence-labeled probes in an Opticon 2 real-time thermocycler (Bio-Rad). cDNA generated from *in vitro* transcribed pure mRNA for each gene was amplified along with the sample and the levels of mRNA were determined from the standard curve.

2.5. Radioimmunoassay of serum gonadotropins and ovarian steroid hormones

FSH, LH, E and P levels in the sera from azaline B-treated hamsters were determined by RIA using previously published protocols (Roy and Greenwald, 1986, 1987, 1989). The sensitivities of P and E assays were 1 ng and 2 pg per tube, respectively. The rat gonadotropin assay antibodies were specific for respective hormones; anti-progesterone antibody had 0.7% cross-reactivity with androstenedione (A), but none with E. The anti-E-antibody did not cross react with either P or A. The levels were presented as pg or ng steroid per ml of serum as appropriate. The interassay and intrassay variations were within 10% and 5%, respectively for each assay.

2.6. Statistical analysis

Ovaries were collected from at least three animals for each group for all experiments. All quantitative data were analyzed by one-way ANOVA with Newman-Keuls *post hoc* test using GraphPad Prism 5 software (Graph Pad software Inc., La Jolla, CA). The level of significance was $p < 0.05$.

3. Results

3.1. Serum levels of FSH, LH, estrogen and progesterone in azaline B-treated hamsters

Levels of P and E in the hamster serum at D4:0900 h had been reported to be 0.6 ± 0.2 ng/ml and 93.3 ± 10.3 pg/ml, respectively (Saidapur and Greenwald, 1978b). Although all hamsters were euthanized on 13th day at 0900 h, which corresponded to day 1:0900 h of the estrous cycle, we used these D4:0900 values as references to compare the changes in serum levels of FSH, LH, P and E in hamsters treated with azaline B and with or without gonadotropins or ovarian steroid hormones. This approach was acceptable because the

ovarian morphology on 13th day was more like D4:0900 h. Whereas ample FSH and LH were present in the serum of D4:0900 h hamsters, FSH was virtually absent (Fig. 1A) and LH (Fig. 1B) was completely undetectable in the serum of azaline B-treated saline or azaline B-treated hormone supplemented hamsters indicating a complete suppression of gonadotropin secretion throughout the experiments. In our hands, Rat-Rat gonadotropin RIAs (National Hormone and peptide Program, A. F. Parlow, Harbor-UCLA Medical Center, CA) did not detect ovine gonadotropins (NIH-20). Whereas marked reduction in serum E and P occurred in azaline B-treated hamsters, FSH and LH significantly upregulated serum E and P levels, respectively. FSH and LH together increased both E and P levels in the serum (Fig. 1C,D).

3.2. Follicular development is altered in hamsters treated with azaline B

Ovaries of azaline B-treated hamsters (Fig. 2A) contained preantral follicles (arrow) as well as many atretic follicles (arrow head), but no antral follicles as expected under gonadotropin deficiency (Greenwald and Terranova, 1984). In contrast to azaline B treated hamsters, FSH replacement resulted in the development of numerous large antral follicles (Fig. 2B, arrows), which reached the preovulatory size (arrows) after LH treatment (Fig. 2D). No antral follicles developed in ovaries exposed to LH (Fig. 2C) or P (Fig. 2F) but there were many preantral (arrows) and some atretic follicles (arrowheads). In contrast, one antral follicle developed in one E-treated ovaries (Fig. 2E and supplemental figure, arrow), and there were several atretic antral follicles (arrowheads). Administration of E plus P resulted in the development of several antral follicles in each ovary (Fig. 2G and supplemental figure, arrows), which was never observed in hypophysectomized hamsters (Roy and Greenwald, 1990; Roy and Kole, 1995).

Consistent with the histological findings, azaline B-treated control hamsters or those treated with LH, E, P or E + P did not exhibit significant changes in the percentage of primordial follicles compared to normal D4 hamsters, but significant decline was evident for FSH and FSH + LH treated groups (Fig. 3A). Such a remarkable recruitment of dormant follicles in the growing pool was not observed in conventional hypophysectomized hamsters treated similarly with FSH or FSH + LH (Moore and Greenwald, 1974). The decline in primordial follicles corresponded to a marked increase in the percentage of primary and preantral follicles, and the formation of antral follicles in FSH treated group (Fig. 3B). In contrast, hamsters treated with FSH + LH had significant decrease in the percentage of preantral follicles with a corresponding remarkable increase in the proportion of antral follicles when compared with the FSH only group (Fig. 3B) indicating an enhanced recruitment of primordial follicles and a rapid development of growing follicles into the antral stage. Whereas gonadal steroid hormone treatment did not cause a statistically significant increase in the percentage of preantral follicles, appreciable increasing trend was evident by 24 h in hamsters treated with P or E + P (Fig. 3B). Several antral follicles developed following E + P administration (Fig. 3B).

3.3. Expression of Cyp11a1, Cyp17a1, Cyp19a1 and FshR mRNA in azaline B treated ovaries

Because gonadotropins and steroid hormones have profound regulatory influence on the steroidogenic enzymes, we wanted to know if ovaries of azaline B-treated hamsters would respond to hormones more efficiently. Consistent with serum steroid hormone levels, mRNA levels of Cyp11a1, Cyp17a1, and Cyp19a1 were decreased in azaline B-treated hamsters (Fig. 4A–C) compared to gonadotropin intact animals (data not shown). Whereas LH markedly induced Cyp11a1 expression, an upward trend was also noted for the FSH-treated group (Fig. 4A). Interestingly, while LH was ineffective, FSH remarkably upregulated the Cyp17a1 mRNA levels (Fig. 4B). CYP17a1 mRNA levels increased further when LH was administered in FSH-primed azaline B-treated hamsters (Fig. 4B). Following estrogen treatment there was a slight, but significant increase in levels of Cyp17a1; however, inclusion of P with E prevented the increase (Fig. 4B). FSH treatment markedly stimulated Cyp19a1 expression with or without LH, but a combined treatment of E and P also augmented ovarian Cyp19a1 mRNA levels consistent with the formation of antral follicles (Fig. 4C). FSH with or without LH stimulated the expression of FshR mRNA, whereas LH alone was ineffective (Fig. 4D). Further, marked increase in FshR expression also occurred in response to E, but the effect was somewhat reduced by P (Fig. 4D).

3.4. Expression of CYP11a1, CYP17a1 and CYP19a1 proteins in azaline B treated ovaries

Immunofluorescence data revealed that increases in steroidogenic enzyme mRNA expression correlated with upregulated enzyme protein expression. Azaline B treatment resulted in a complete downregulation of CYP11a1 and CYP17a1 in theca as well as in the interstitial cells (Figs 5A, 6A). The absence of antral follicles coincided with low basal CYP19a1 expression (Fig. 7). Whereas FSH had no effect (Fig. 5B), a modest increase in CYP11a1 protein expression in the interstitial cells was noted in response to LH alone (Fig. 5C). FSH with LH markedly enhanced CYP11a1 expression in the interstitial and theca cells as well as in the mural granulosa cells of large antral follicles (Fig. 5D). E induced very low CYP11a1 expression in thecal cells (Fig. 5E). Low but a noticeable increase in CYP11a1 expression was evident in the interstitial and theca cells exposed to P (Fig. 5F) with or without E (data not shown).

In azaline treated ovaries, CYP17a1 expression was detectable in few cells of the theca (Fig. 6A). Whereas treatment with LH induced discrete CYP17a1 expression in several thecal cells of preantral follicles (Fig. 6C), remarkable expression was evident in ovaries exposed to FSH *in vivo* (Fig. 6B). FSH + LH treatment was also effective in inducing CYP17a1 expression in thecal cells of antral follicles (Fig. 6D). Whereas E induced CYP17a1 in few thecal cells of small antral follicles, marked expression was evident in the theca of large antral follicles (Fig. 6E). The expression pattern remained unaltered when P was administered along with E (Fig. 6F), but P alone had no effect (Fig. 6G).

As expected, immunofluorescence data confirmed that CYP19a1 expression was limited in the granulosa cells of the preantral and antral follicles (Fig. 7). No appreciable CYP19a1 expression was noted without gonadotropin in azaline treated hamsters (Fig. 7A); however, FSH treatment resulted in a marked expression of CYP19a1 in the mural granulosa cells of

antral follicles and in the granulosa cells of preantral follicles (Fig. 7B). Whereas LH alone was ineffective (Fig. 7C), a combination of FSH and LH further augmented CYP19a1 expression in the granulosa cells of large preantral and antral follicles (Fig. 7D). Interestingly, E alone induced appreciable CYP19 expression in the granulosa of preantral (Fig. 7E, top) and one antral follicles (Fig. 7E, bottom) and the expression increased markedly when P was administered along with E (Fig. 7F). However, P alone had no effect (Fig. 7G).

4. Discussion

Gonadotropin regulation of ovarian steroidogenic enzymes has been well established in many species using hormonal replacements in immature or hypophysectomized animals (Gore-Langton and Armstrong, 1994; Greenwald and Roy, 1994). We wanted to know if selective removal of gonadotropins without altering the secretion of other important pituitary tropic hormones would present an efficient physiological milieu to examine the role of gonadotropins in ovarian follicular development and functions, especially in the golden hamster. The results of this study suggest that azaline B as a GnRH antagonist completely blocks gonadotropin secretion resulting in not only an arrest in preantral to antral follicle transition, but also a significant reduction in the entry of primordial follicles into the growing pool, the development of preantral follicles, and ovarian steroidogenesis. Azaline B has been shown to act as a potent GnRH antagonist in rats and remains effective for at least 48 h (Rivier et al., 1995b; Samant et al., 2005). Although serum prolactin levels have not been measured in the present study because azaline B is not expected to directly affect pituitary prolactin secretion, a decrease in ovarian E production in the absence of FSH may indirectly reduce pituitary prolactin secretion. However, prolactin has been shown to exert a luteotropic effect along with FSH in pregnant hamsters (Greenwald, 1986), but it does not promote follicle development in hypophysectomized hamsters (Kim and Greenwald, 1984). Further, administration of prolactin in hypophysectomized rats suppresses ovarian 3β -hydroxysteroid dehydrogenase expression (Martel et al., 1994), and experimentally induced hyperprolactinemia in rats causes ovarian cyst formation, higher serum E, but inhibits CYP17a1 and CYP19a1 activities (Munabi et al., 1984). Therefore, none of the observed ovarian changes can be ascribed to the lack or increase in prolactin secretion, if any, after E administration. A modest decrease in preantral follicle number and the formation of few antral follicles following higher doses of FSH have been shown in hypophysectomized hamsters (Moore and Greenwald, 1974). However, the marked development of primary and preantral follicles, and the development of several large antral follicles in each ovary following a relatively low dose of FSH suggest that follicular cells are maintained in otherwise healthy state in azaline B-treated hamsters whereby more viable follicles are available to respond to FSH. A rapid progression of preantral follicles to antral stage in the hamster ovary following unilateral ovariectomy has been reported (Chiras and Greenwald, 1978). Although levels of ovine FSH remaining in the blood 16 h after the last FSH injection has not been determined in the present study, earlier studies on hamsters have shown that approximately 0.036% of FSH remains in the blood 4 days after a sc injection of 5 μ g of a less pure NIH ovine FSH-16 (Kim and Greenwald, 1986). Further, sc administration of 60 μ g/kg/day FSH to C57BL/6J mice for 30 days results in a serum FSH level approximately

35 ng/ml (Ritter et al., 2008). Therefore, 10 µg ovine-FSH, which is selected based on many published reports and studies done in our laboratory, is expected to maintain serum FSH levels at low physiological limit. The increase in serum E following FSH treatment also highlights the functional development of antral follicles. Such a remarkable increase in serum E following FSH has not been observed in hypophysectomized hamsters treated with FSH (Roy, 2000). The administration of LH to FSH-treated hamsters to mimic the normal LH surge promotes preovulatory follicle development in number as well as in CYP19a1 expression. The results offer the first direct evidence that under appropriate endocrine milieu, FSH activates the entry of primordial follicles into the primary stage in hamsters, thus confirming our earlier findings in neonatal hamsters (Roy and Albee, 2000). The results also highlight that non-gonadotropic pituitary hormones are important to maintain the basic metabolic functions of follicular cells.

Although deletion of the *Fshβ* gene in mice also results in functional FSH deficiency and arrest in follicular development at the preantral stage (Kumar et al., 1997), the approach is limited to the mouse; more specifically to one strain of mouse. *Fsh*-receptor null mutation phenocopies *Fshβ* knockout (Dierich et al., 1998), but the model does not allow FSH replacement studies. Whereas hypophysectomy has been the only option for other laboratory animals, the endocrine compromised environment may affect the sensitivity of ovarian cells to gonadotropins or ovarian steroids. Such a possibility may explain the superior response to hormones observed in azaline B-treated hamsters. Azaline B treatment also eliminates the surgical stress, which may adversely affect follicular functions in the long run, thus compromising follicular response to gonadotropins. Evidence suggests that T3 enhances follicular response to FSH leading to increased development of preantral follicles in mice (Zhang et al., 2013). T3 also prevents granulosa cell apoptosis following chemotherapy (Verga Falzacappa et al., 2012). Similarly, Sasson and Amsterdam have shown that dexamethasone prevents granulosa cells apoptosis by inducing antiapoptotic *Bcl2* gene and suppressing the synthesis and activity of granzyme B, an apoptosis inducer protease (Sasson and Amsterdam, 2003b). One of the most notable findings is that E along with P not only promotes primary follicle formation, but also stimulates the formation of healthy antral follicles with appreciable CYP19a1 expression. The increase in *Cyp19a1* mRNA correlates well with CYP19a1 protein expression in antral follicles that have developed following E + P treatment. E + P does not induce similar effect in long-term hypophysectomized hamsters (unpublished observation). Wang and Greenwald (1993) have shown that hypophysectomized ICR mice treated for 4 days with 10–250 µg/day estradiol cyclopropionate (ECP) have significantly large number of preantral follicles, but even the highest dose of ECP fails to induce antral follicle formation. A high dose of E has been shown to induce preantral follicles in immature intact or hypophysectomized rats (Richards et al., 1976b). E has been shown to upregulate FSH receptors in ovarian granulosa cells and to affect ovarian folliculogenesis in many species (Louv et al., 1976; Palter et al., 2001). In hypophysectomized immature rats, E fails to induce FSH-receptor within 24 h (Ireland and Richards, 1978), but it can induce FSH-receptor after 4 days of treatment (Richards et al., 1976b). The stimulatory effect of P on preantral follicle development has been reported in hypophysectomized hamsters (Moore and Greenwald, 1974), but P seems to have minor effect on ovarian steroidogenic enzymes.

Increased expression of CYP17a1 in the theca by FSH is paradoxical considering that thecal cells do not express FSH receptors (Oxberry and Greenwald, 1982), and it has been shown in almost all mammals that LH regulates thecal CYP17a1 expression (Gore-Langton and Armstrong, 1994). Therefore, it stands to reason that ovarian CYP17a1 expression in azaline + FSH-treated hamsters is due to LH contamination, albeit extremely low (50 pg/ng by radio-immunoassay) in ovine-FSH-20 preparation. However, unaltered levels of ovarian Cyp17a1 mRNA and modest expression of CYP17a1 protein in hamsters treated with 5 µg ovine-LH-25 also suggest that thecal CYP17a1 expression requires the presence of appropriate levels of LH-receptors, which in turn depends on FSH-induced follicular development. Induction of CYP17a1 expression in thecal cells has been shown to depend on a synergism between FSH and LH (Okuda et al., 1997). Alternatively, granulosa cells developed under FSH may produce paracrine factors that upregulate CYP17a1 expression in the theca. In fact, exposure of immature rat ovaries *in vivo* or granulosa-theca cells co-cultures *in vitro* to FSH for 24 h results in marked increases in ovarian or thecal CYP17a1 mRNA expression, and the effect can be blocked by antibodies to inhibin-α or β-glycan (Hoang et al., 2013). The increased expression of ovarian CYP17a1 protein in thecal and interstitial cells, and in mural granulosa cells of large antral follicles after FSH + LH administration also supports our conjecture that sensitivity to LH depends on follicular development. Thecal cells in hypophysectomized hamsters respond poorly to LH (Garnett et al., 2002). The robust expression of Cyp11a1, Cyp17a1, Cyp19a1 mRNA and proteins, and FshR mRNA in response to FSH + LH suggests that in contrast to hypophysectomized hamsters, follicular response to gonadotropins is better preserved in azaline B treated pituitary intact hamsters.

In summary, the results of this study suggest that pituitary gonadotropin secretion can be effectively blocked by azaline B, a GnRH antagonist, which preserves the secretion of other pituitary hormones. This selective removal of gonadotropin effects without altering the functions of other metabolic hormones regulated by TSH, ACTH or GH appears to maintain the granulosa and thecal cells in physiological conditions even though follicular growth ceases due to gonadotropin deficiencies. Such an otherwise intact endocrine environment seems to be essential to unveil the true effect of FSH on folliculogenesis from primordial through antral follicles in the hamsters. Azaline B approach contrasts with that of conventional hypophysectomy, which eliminates the support of non-gonadotropic hormones, thus creating endocrine deficiencies, which is likely to adversely affect all cells in the body including the ovary. Whereas selective gonadotropin deficiency can be achieved with null mutation, the broader limitation of this approach makes azaline B a suitable alternative to study selectively the effect of gonadotropins on ovarian functions in other non-mouse animal species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FSH	Follicle stimulating hormone
LH	Luteinizing hormone
E	Estradiol 17 β
P	Progesterone
GnRH	Gonadotropin releasing hormone
RIA	Radioimmunoassay

Appendix: Supplementary material

Supplementary data to this article can be found online at [doi:10.1016/j.mce.2014.11.011](https://doi.org/10.1016/j.mce.2014.11.011)

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Fig. 1. Serum levels of (A) progesterone (P) and (B) estradiol-17 β (E) in hamsters treated with azaline B (control, C) and with or without gonadotropins or steroid hormones. Each bar is equal to mean $\pm$ SEM. $P < 0.05$ = bars with different letters.

Fig. 2.

Assembled H & E stained histological pictures of whole ovaries of hamsters treated with (A) azaline B, and with (B) 20 μ g ovine-FSH-20 on 11th and 12th days of azaline B treatment (arrows indicate antral follicles), (C) 5 μ g ovine-LH-25 at 1400 h on 12th day of azaline treatment (arrow indicates preantral follicles and arrowheads indicate atretic follicles), (D) 20 μ g ovine-FSH-20 on 11th and 12th days and 5 μ g ovine-LH-25 at 1400 h on 12th day of azaline treatment (arrows indicate antral follicles), (E) 0.1mg E at 0900 h on 12th day of azaline treatment (arrow indicates antral follicle and arrowheads indicate atretic follicles), (F) 0.5mg P at 0900 h on 12th day of azaline treatment (arrows indicate preantral follicles) or (G) 0.1 mg E and 0.5 mg P at 0900 h on 12th day of azaline treatment (arrows indicate antral follicles). Bar = 1000 μ m.

Fig. 3. Development of primordial (A) or primary through antral stages (B) follicles in ovaries of azaline B-treated hamsters (control, C) exposed in vivo to FSH, LH, E or P. Each bar represents the mean $\pm$ SEM. $P < 0.05$ = bars with different letters.

Fig. 4. Levels of Cyp11a1 (A), Cyp17a1 (B), Cyp19a1 (C) and FshR (D) mRNA in total ovarian RNA of hamsters treated with azaline B (control) with or without FSH, LH, E or P. Each bar represents the mean $\pm$ SEM of the ratio of respective enzyme and β -actin mRNA. $P < 0.05$ = bars with different letters.

Fig. 5.

Immunofluorescence localization of CYP11a1 protein in the ovaries of hamsters treated with azaline B (A) and with FSH (B), LH (C), FSH + LH (D), E (E) or P (F). GC, granulosa cells, Th, thecal cells, IC, interstitial cells, aGC, antral granulosa cells, mGC, mural granulosa cells. CYP11a1, green fluorescence, nuclei, DAPI, red pseudo color for image contrast. Bar = 10 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Immunofluorescence localization of CYP17a1 protein in the ovaries of hamsters treated with azaline B (A) and with FSH (B), LH (C), FSH + LH (D), E (E), E + P (F) or P (G). GC, granulosa cells, Th, thecal cells, IC, interstitial cells. CYP17a1, green fluorescence, nuclei, DAPI, red pseudo color for image contrast. Bar = 10 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Immunofluorescence localization of CYP19a1 protein in the ovaries of hamsters treated with azaline B (A) and with FSH (B), LH (C), FSH + LH (D), E (E; top, preantral, bottom, antral follicles), E + P (F) or P (G). GC, granulosa cells, Th, thecal cells, IC, interstitial cells, PA, preantral follicles. CYP19a1, green fluorescence, nuclei, DAPI, red pseudo color for image contrast. Bar = 10 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)