Letrozole increases ovarian growth and *Cyp17a1* gene expression in the rat ovary

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Objective: To evaluate the effects of letrozole on ovarian size and steroidogenesis in vivo, as well as on proliferation and steroidogenesis of theca-interstitial cells alone and in coculture with granulosa cells using an in vitro model.
Design: In vivo and in vitro studies.
Setting: Research laboratory.
Animal(s): Immature Sprague-Dawley female rats.
Intervention(s): In vivo effects of letrozole were studied in intact rats receiving either letrozole (90-day continuous-release SC pellets, 400 μg/d) or placebo pellets (control group). In in vitro experiments, theca cells were cultured alone or in coculture with granulosa cells in the absence or presence of letrozole.
Main Outcome Measure(s): Deoxyribonucleic acid synthesis was determined by thymidine incorporation assay; steroidogenesis by mass spectrometry; and steroidogenic enzyme messenger RNA (mRNA) expression by polymerase chain reaction.
Result(s): In vivo, letrozole induced an increase in ovarian size compared with the control group and also induced a profound increase of androgen, LH levels, and *Cyp17a1* mRNA expression. Conversely, a decrease in *Star*, *Cyp11a1*, and *Hsd3b1* transcripts was observed in letrozole-exposed rats. In vitro, letrozole did not alter either theca cell proliferation or *Cyp17a1* mRNA expression. Similarly, letrozole did not affect *Cyp17a1* transcripts in granulosa-theca cocultures.
Conclusion(s): These findings suggest that letrozole exerts potent, but indirect, effect on growth of rat ovary and dramatically increases androgen levels and *Cyp17a1* mRNA expression, the key enzyme regulating the androgen biosynthesis pathway. The present findings reveal novel mechanisms of action of letrozole in the rat ovary. (Fertil Steril® 2013;99:889–96. ©2013 by American Society for Reproductive Medicine.)
Key Words: CYP17A1, letrozole, ovarian theca-interstitial cells, proliferation, steroidogenesis

Aromatase is a cytochrome P450 enzyme responsible for the rate-limiting step in estrogen biosynthesis, catalyzing the conversion of C19 steroids, androstenedione (A), and T into C18 steroids, estrone, and E_2, respectively (1). Aromatase, encoded by *CYP19*, is widely distributed in several cells, including the ovarian granulosa cell, the placental syncytiotrophoblast, and the testicular Leydig cells, as well as various extraglandular sites such as brain, fat, and skin (2). Letrozole is a potent and highly specific nonsteroidal aromatase inhibitor that competitively binds to the heme of the cytochrome P450 subunit, leading to a near-complete blockade of the aromatization in peripheral tissues without exerting effects on other steroidogenic pathways (3).

Since its approval as first-line therapy for hormone receptor–positive, metastatic breast cancer in postmenopausal women in 1997 (4), letrozole has emerged as a promising therapeutic agent to ameliorate certain gynecologic disorders, expanding its indications for use in premenopausal women. Recent studies

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have demonstrated the ovulation-inducing capacity of letrozole (5, 6), showing potential advantages over clomiphene citrate, including the development of a monofollicular response and the lack of adverse effects on either the endometrium or the cervical mucus (7, 8). In addition, a letrozole-induced hypoestrogenic effect may play a role in the treatment of some estrogen-dependent diseases in premenopausal women, such as endometriosis and uterine fibroids (9, 10).

However, little is known about the potential effect of letrozole on the regulation of ovarian growth and steroidogenesis. Manneras et al. (11) demonstrated an increase in ovarian size and number of cystic follicles together with theca-interstitial cell hyperplasia in letrozole-exposed adult female rats, meeting the morphologic criteria for polycystic ovary syndrome. In contrast, Kafali et al. (12) did not detect any changes in ovarian weight after letrozole treatment. Furthermore, the role of letrozole in the regulation of the key genes involved in steroidogenesis needs to be evaluated. Immunohistochemical studies have demonstrated an increase in the expression of the androgen receptor, steroidogenic acute regulatory protein (Star), and 3-β-hydroxysteroid dehydrogenase (Hsd3b1) and a decrease in estrogen receptor β in letrozole-induced polycystic ovaries of rats (13). However, it is not known whether letrozole affects expression of the genes involved in the ovarian androgen biosynthesis pathway, such as *Cyp17a1*. Furthermore, it is not known whether altered ovarian growth and androgen production are due to local–intraovarian effects of letrozole, such as reduced aromatization of androgens on the ovarian level, or due to altered hypothalamo–pituitary function resulting in altered release of LH.

In view of these considerations, the present study was designed to evaluate the potential effect of letrozole on ovarian growth and steroidogenesis using both in vivo and in vitro rodent models.

MATERIALS AND METHODS

Animals

Three Wistar dams, each with 10 female pups, were obtained from Charles River Laboratories. Pups were raised with the lactating dam (not the biological mother of all the pups) until 21 days of age and then housed two per cage under controlled conditions (21–24°C, 55%–65% humidity, 12-hour light/12-hour dark cycle). Rats were fed standard commercial food and tap water ad libitum. At 21 days of age, rats were randomly divided into two experimental groups (control [n = 14] and letrozole [n = 14]) and implanted SC with 70-day continuous-release pellets (Innovative Research of America) containing 28 mg of letrozole (daily dose, 400 μg) (Novartis Pharma) or placebo. The dose of letrozole was chosen according to a previous study (12). The control group received identical pellets lacking the bioactive molecule. The animals were anesthetized using isoflurane before the SC insertion of the pellet. Rats were weighed every week from 21 days of age. Estrous cycle stage was determined microscopically with Giemsa staining of the predominant cell type in vaginal smears obtained daily from the ninth week of age to the end of the experiment, considering cycles with duration of 4 to 5 days to be regular (14, 15). The study was concluded after 10 weeks of exposure to letrozole, when the rats were 13 weeks of age. All treatments and procedures were carried out in accord with accepted standards of human animal care as outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and a protocol approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

Tissue Sampling

After 10 weeks the rats were killed in diestrous phase by intracardiac perfusion of 0.9% saline under anesthesia using ketamine and xylazine (75/5 mg/kg, IP); trunk blood was collected, and plasma was stored at −20°C until assayed. The heart, liver, pancreas, adrenals, spleen, kidneys, uterus, and ovaries were excised. One half ovary from each animal was frozen and stored at −80°C for subsequent use.

Ovarian Morphology

One ovary from each animal was fixed in 10% formalin, embedded in paraffin wax, and then sectioned serially at 5-μm thickness. Sequential sections were mounted and stained by the hematoxylin and eosin procedure. The specimens were evaluated under ×400 magnification.

Total RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from the ovarian specimen and from theca-interstitial cells using the MagMAX-96 Total RNA Isolation Kit (Applied Biosystems) and the KingFisher robot (Thermo Scientific). Reverse transcription of total RNA to complementary DNA (cDNA) was performed using the High Capacity cDNA Reverse Transcription Kit for reverse transcription–polymerase chain reaction (RT-PCR) (Applied Biosystems). The PCR assays were set up in 28-μL volumes, consisting of 5 μL cDNA, 4.5 μL forward and 4.5 μL reverse 900 nM primers, and 14 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems).

Quantitative real-time PCR reactions were performed in triplicate using the ABI 7300 Real-Time PCR System (Applied Biosystems). Separate cDNA dilutions were included in each PCR run to generate standard curves. Data were analyzed using SDS 1.4 software (Applied Biosystems). The relative amount of target messenger RNA (mRNA) was expressed as a ratio normalized to hypoxanthine phosphoribosyltransferase (*Hprt*). The primer sequences were as follows: rat *Star* forward (5′-GCC TGA GCA AAG CGG TGT C-3′) and reverse (5′-CTG GCG AAC TCT ATC TGG GTC TGT-3′); rat *Cyp11a1* forward (5′-GCT GGA AGG TGT AGC TCA GG-3′) and reverse (5′-CAC TGG TGT GGA ACA TCT GG-3′); rat *Hsd3b1* forward (5′-CCA GAA ACC AAG GAG GAA T-3′) and reverse (5′-CCA GAA ACC AAG GAG GAA T-3′); rat *Cyp17a1* forward (5′-ACT GAG GGT ATC GTG GAT GC-3′) and reverse (5′-CCG TCA GGC TGG AGA TAG AC-3′); and rat *Hprt* forward (5′-TTG TTG GAT ATG CCC TTG ACT-3′) and reverse (5′-CCG CTG TCT TTT AGG CTT TG-3′).

Sample Preparation and Processing for Quantification of Steroids

Androstenedione and T were obtained from Steraloids, whereas testosterone-d3 was obtained from Cerillient. Acetonitrile and