Inhibition of androgen and oestrogen production by clomiphene citrate in avian theca cells

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Summary. Isolated theca cells (2×10^5 /ml) were pre-incubated for 1 h in the presence or absence of clomiphene citrate (10^{-12} – 10^{-4} M). Ovine LH (50 ng/ml) was added and cells were incubated for an additional 3 h. A 50% inhibition of LH-stimulated androstenedione and oestrogen production was obtained with doses of 10^{-8} M and 2×10^{-7} M clomiphene, respectively. Furthermore, the effect of clomiphene on LH-stimulated androstenedione production was reversed by washing clomiphene from the cells before stimulation with LH. In subsequent experiments, the effects of clomiphene on C17–20-lyase and aromatase activities were examined. Conversion of [3 H]17-hydroxyprogesterone to androstenedione was inhibited by 50% when theca cells were pretreated with 10^{-5} M-clomiphene. In addition, conversion of testosterone to oestrogen by theca cells was inhibited in a dose-dependent manner by clomiphene, with 50% inhibition occurring at a dose of 5×10^{-6} M. The results show that clomiphene treatment *in vitro* inhibits androgen and oestrogen production in theca cells by inhibitory effects on the activities of C17–20-lyase and aromatase. In addition to the widely-accepted effects of clomiphene on the hypothalamic–pituitary axis, the present findings add further support to the suggestion that clomiphene exerts direct effects on ovarian steroidogenesis.

Introduction

Clomiphene citrate is one of the drugs most commonly used to induce ovulation in anovulatory women. Clinical studies have demonstrated that clomiphene induces ovulation in 80% of anovulatory women, but results in only a 40% pregnancy rate (Speroff *et al.*, 1983). The discrepancy between rates of ovulation and pregnancy has led investigators to examine the effects of clomiphene more intensely. It is generally accepted that clomiphene exhibits oestrogenic and antioestrogenic effects and induces ovulation in a complex fashion. There are believed to be 3 sites of action (Adashi, 1984). The primary site is the hypothalamus, where clomiphene blocks the negative feedback of oestrogen, thereby enhancing release of gonadotrophins. Secondary sites are the pituitary, where it enhances the GnRH-stimulated release of FSH, and the ovary, where its effects are not completely clarified.

Clomiphene inhibits progesterone synthesis in rat follicles (Laufer *et al.*, 1982) and granulosa cells (Welsh *et al.*, 1984), monkey luteal cells (Westfahl & Resko, 1983) and hen granulosa cells (Sgarlata *et al.*, 1984). In contrast, the effects of clomiphene on oestrogen biosynthesis *in vitro* have been less consistent. Some investigators report a stimulation (Zhuang *et al.*, 1982; Welsh *et al.*, 1984), and others an inhibition (Laufer *et al.*, 1982). The effect of clomiphene on androgen synthesis has not been reported.

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In an effort to understand further the actions of clomiphene on ovarian steroidogenesis, we investigated the in-vitro effects of clomiphene on androgen and oestrogen production by theca cells from maturing ovarian follicles of the domestic hen. The domestic hen has only one functional ovary and ovulates daily for several consecutive days. The maturing yolk-filled follicles are arranged in a size hierarchy according to proximity to ovulation. Production of oestrogen and androgen by the preovulatory follicles occurs exclusively in the theca compartment and is influenced by LH and by the stage of follicular development. Furthermore, these cells contain C17-20-lyase (Marrone & Hertelendy, 1985) and aromatase (Armstrong, 1984) activities. The aromatase and C17-20-lyase activities are greatest in theca cells from the follicles that are 3 and 4 days away from ovulation (Armstrong, 1984; Marrone & Hertelendy, 1983a, b, 1985).

Materials and Methods

Experimental animals

One- to two-year-old White Leghorn hens were obtained from the University of Missouri-Columbia or from Ralston Purina Farms, Gray Summit, MO. Birds selected for use in this study had regular clutches of 5 or more eggs. The time of oviposition was recorded by an automatic device. The birds were caged individually in a windowless room and kept under a 14 h light/10 h dark cycle. Purina Layena ration and water were provided *ad libitum*.

Hormones and chemicals

Ovine LH (NIADDKD-LH-22; 2.3 NIH-LH-S1 U/mg) was a gift from the National Hormone and Pituitary Program of the National Institutes of Health. All steroids, the bovine serum albumin, soybean trypsin inhibitor, 3-isobutyl-1-methylxanthine (IBMX), and clomiphene citrate (Clomid, 67% cis, 33% trans) were purchased from Sigma Chemical Co. (St Louis, MO). Powdered Medium 199 with Hanks' salts was purchased from Grand Island Biological Co. (New York, NY). [1,2-³H]17-Hydroxyprogesterone (sp. act. 40–60 Ci/mmol), [2,4,6,7-³H]oestradiol-17 β (sp. act. 90–115 Ci/mmol), and [1,2,6,7-³H]androstenedione (sp. act. 80–115 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA). Baker and Fisher high-performance liquid chromatography (HPLC) solvents were used.

Tissue collection and cell preparation

Birds were killed by cervical dislocation 1–4 h before expected ovulation, as in previous experiments (Marrone & Hertelendy, 1983a, b, 1985). Theca cells were obtained from the third and fourth largest follicles and isolated by collagenase digestion as described previously (Marrone & Hertelendy, 1983a). Viability of the theca cell preparations, as determined by exclusion of 0.01% trypan blue, was routinely greater than 90%. Cells in each preparation were counted with the aid of haemocytometer and each cell preparation was diluted to a final concentration of 1×10^6 viable cells/ml Medium 199.

Incubations

Incubations were carried out as described previously (Marrone & Hertelendy, 1983a). For each cell suspension, 200 μ l containing 2×10^5 viable cells were pipetted at 4°C into 12 $\times$ 75 mm polystyrene culture tubes that contained Medium 199 and various doses of clomiphene citrate. When LH was used, 0.1 mM-3-isobutyl-1-methylxanthine (IBMX) was added to the incubation at 0 h to

inhibit phosphodiesterase activity. After a 1 h preincubation, the treatment hormone (10 ng LH, 250 ng testosterone, or 250 ng [^3H]17-hydroxyprogesterone) was added, making a total volume of 1 ml in each tube, and cells were incubated for an additional 3 h.

There were 5 replicate incubation tubes from the same cell preparation per treatment group in each experiment. Incubations were done at 37°C in a shaking water bath under air for 3 h and were terminated by placing the tubes in an ice bath. The incubation tubes were frozen until extraction for radioimmunoassay or HPLC.

Radioimmunoassay

The incubation media and cells were extracted twice with 2 volumes of diethyl ether. The extraction efficiencies for oestrogen and androstenedione were both >95%. The extract was evaporated to dryness and redissolved in ethyl alcohol; 75% of the sample was taken for oestrogen radioimmunoassay and 2–10% was taken for androstenedione radioimmunoassay.

Details of the radioimmunoassays for oestrogens and androstenedione have been reported previously (Marrone & Hertelendy, 1985). The oestrogen antiserum (provided by Dr B. V. Caldwell, Yale University) reacts equally well with oestradiol-17 β and oestrone and has a 10% cross-reactivity with oestriol. The inter- and intra-assay coefficients of variation were determined to be 11% and 5%, respectively, and the sensitivity was 10 pg per tube. The androstenedione antiserum (ANS-22 from Endocrine Sciences, Tarzana, CA) cross-reacts with 1,4-androstadiene-3,17-dione (40%), 5 α - and 5 β -androstane-3,17-dione (35%), androsterone and epiandrosterone (4%), androstane (3.5%), 5 α -androstane-3,11,17-trione (2.5%), and testosterone (2%). The coefficients for inter- and intra-assay variation were both 10–11% and the sensitivity of the assay was 5 pg per tube. Samples from each experiment were analysed in the same radioimmunoassay.

HPLC

Samples were extracted with diethyl ether as above and evaporated to dryness under a gentle stream of nitrogen in a 45°C water bath. The extraction efficiencies of the radioactivity were routinely >95%. The sample was dissolved in 25 μl HPLC-grade methanol and 20 μl were injected into a 5 μm reversed-phase C₁₈ column (Altex Ultrasphere, 15 cm $\times$ 4.6 mm). The mobile phase was tetrahydrofuran : methanol : water (16 : 28 : 56 by vol.) at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected for scintillation counting and the profile was visualized by u.v. spectroscopy at 240 nm. Fractions were collected for 30 min and the total radioactivity in 60 fractions was calculated. All of the metabolites of [^3H]17-hydroxyprogesterone are eluted within this period. Determination of the production of each steroid metabolite was based on co-elution of radioactivity with a known steroid standard. The radioactivity in each peak was expressed as a percentage of the total radioactivity. Only fractions that had radioactivity twice that of the background level were used for the calculations.

Details of the equipment and separation profile of this solvent system have been reported previously (Marrone & Hertelendy, 1983b, 1985; Marrone *et al.*, 1985).

Data analysis

Comparisons between two groups were analysed by Student's *t*-test. Comparisons among more than two groups were analysed by 1-way analysis of variance and Neuman-Keuls post-hoc tests. Probability values of <0.05 were considered statistically significant.

Results

Baseline androstenedione and oestrogen production by theca cells was increased significantly in

Fig. 1. Dose-response curve of the inhibitory effect of increasing doses of clomiphene on LH-stimulated (open bars and open triangles) and basal production (hatched bar and solid triangles) of androstenedione (top panel) and oestrogen (bottom panel). Theca cells were pre-treated for 1 h with clomiphene, then incubated for 3 h after the addition of LH (50 ng/ml) or 100 μ l media. The values represent the mean $\pm$ s.e.m. from 3–4 separate experiments.

response to ovine LH (50 ng/ml). When theca cells were preincubated for 1 h with clomiphene citrate an inhibition of the LH-stimulated androstenedione and oestrogen production was observed (Fig. 1). Clomiphene demonstrated a dose-dependent inhibition of LH-stimulated androstenedione production which was significant statistically from doses of 10^{-8} to 10^{-4} M-clomiphene. A 50% inhibition of LH-stimulated androstenedione production was obtained with a dose of $\sim 10^{-8}$ M-clomiphene. There also appeared to be a dose-dependent inhibition of oestrogen production. A significant inhibition of oestrogen production occurred at doses of 10^{-5} and 10^{-4} M-clomiphene. A 50% inhibition of LH-stimulated oestrogen production was obtained at a dose of approximately 2×10^{-7} M-clomiphene. Basal (unstimulated) production of androstenedione and oestrogen was not consistently influenced by clomiphene.

In an effort to determine which enzymes were inhibited by clomiphene, we examined the effects of clomiphene on conversion of [³H]17-hydroxyprogesterone to androstenedione, as a measure of C17–20-lyase activity. Theca cells that had been preincubated with or without 10^{-5} M clomiphene were treated with 250 ng [³H]17-hydroxyprogesterone. The major metabolites of 17-hydroxyprogesterone, as determined previously (Marrone & Hertelendy, 1985; Marrone *et al.*, 1985), are androstenedione and 17 α ,20 β -dihydroxy-4-pregnen-3-one. The peaks of radioactivity represented

by the substrate, androstenedione and $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one in 5 experiments comprised $94 \pm 0.8\%$ of the total radioactivity collected in 60 fractions (30 min). The remaining 5% of the total radioactivity was distributed in the fractions that eluted before, between, and after these 3 peaks. Theca cells preincubated with clomiphene showed an inhibition of $[^3\text{H}]17$ -hydroxyprogesterone conversion (Fig. 2). Androstenedione production was significantly reduced in those

Fig. 2. Effect of clomiphene citrate on conversion of 17-hydroxyprogesterone (17OH) to androstenedione (ΔA) and $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ($17\alpha20\beta$). Theca cells (2×10^5) were pretreated for 1 h with 10^{-5} M-clomiphene (hatched bars) or without clomiphene (open bars). $[^3\text{H}]17\text{OH}$ (250 ng/ml) was added and cells were incubated for an additional 3 h. Steroids were separated by HPLC and the radioactivity in each peak was expressed as the percentage of the total recovered radioactivity. Each bar represents the mean $\pm$ s.e.m. from 5 separate experiments. * $P < 0.05$; ** $P < 0.01$ compared with value without clomiphene.

Fig. 3. Dose-response curve of the inhibitory effect of increasing doses of clomiphene on conversion of testosterone to oestrogen. Theca cells were pretreated for 1 h with clomiphene, then incubated for 3 h after the addition of testosterone (250 ng/ml). The values represent the mean $\pm$ s.e.m. of 4-6 separate experiments.

cells treated with clomiphene when compared to the control ($P < 0.05$). In addition, a significantly greater amount of [^3H]17-hydroxyprogesterone remained in clomiphene-pretreated cells ($P < 0.01$). Although there appeared to be a reduction in the formation of 17 α ,20 β -dihydroxy-4-pregnen-3-one, this reduction was not statistically significant.

We next examined the effect of clomiphene on the conversion of testosterone to oestrogen by theca cells, an index of aromatase activity. Armstrong (1984) has shown that oestradiol-17 β is the only product detected after incubation of hen thecal homogenates with tritiated testosterone. In theca cells pretreated with clomiphene, and then incubated with 250 ng testosterone, conversion of testosterone to oestrogen was inhibited (Fig. 3). Oestrogen production rose significantly after incubation with testosterone. There was no change from control with doses of 10^{-12} to 10^{-9} M-clomiphene, but a dose-dependent inhibition occurred between 10^{-7} and 10^{-4} M. Testosterone conversion was significantly inhibited (by 80%) at 10^{-4} M-clomiphene and, when baseline oestrogen values were subtracted, 50% inhibition occurred at a dose of 5×10^{-6} M-clomiphene.

To assess whether the inhibitory effects of clomiphene could be reversed, theca cells were preincubated with 10^{-5} M-clomiphene for 4 h, washed three times, and then incubated with or without 50 ng LH/ml for 3 h in the absence of clomiphene. There were no significant differences in androstenedione production between the clomiphene-pretreated or non-pretreated groups after LH treatment. The mean androstenedione values (pg/ 2×10^5 cells/ml; mean $\pm$ s.e.m.) of 4 separate experiments were: no clomiphene pretreatment, without LH (98.2 ± 45.3) or with LH (451 ± 68.0); clomiphene pretreatment, without LH (49.8 ± 20.9) or with LH (360 ± 74.4).

Discussion

This study provides evidence that clomiphene inhibits androstenedione and oestrogen production in hen theca cells *in vitro*. Clomiphene inhibited LH-stimulated androstenedione and oestrogen production in a dose-dependent manner and, in addition, inhibited both the aromatase and C17-20-lyase enzyme activities. Furthermore, the inhibitory effects of clomiphene on steroidogenesis were reversible. An inhibitory effect of clomiphene on the aromatase activity would obviously result in reduced oestrogen production. Likewise, an inhibitory effect of clomiphene on C17-20-lyase activity could contribute indirectly to a reduction in oestrogen production, by limiting the availability of androgen substrate for aromatization to oestrogen.

The inhibitory effects of clomiphene on ovarian steroidogenesis may have two consequences. First, the reduced oestrogen production may enhance LH secretion. It is believed that clomiphene acts at the level of the hypothalamus to displace oestrogen from its receptors, thereby preventing the negative feedback of oestrogen on gonadotrophin release. As a result, LH secretion is stimulated. The hypothalamic action of clomiphene could be enhanced by a direct inhibitory effect of clomiphene on oestrogen production. On the other hand, impaired folliculogenesis may be another consequence of a direct inhibitory effect of clomiphene on ovarian steroidogenesis. Appropriate quantities of oestrogen are needed, with gonadotrophins, to stimulate follicular maturation (see Goodman & Hodgen, 1983). Therefore, an inhibitory effect of clomiphene on oestrogen biosynthesis could impair the process of folliculogenesis.

There are conflicting reports concerning the effects of clomiphene on oestrogen production. Laufer *et al.* (1982) reported an inhibition of oestrogen production by clomiphene *in vitro* by rat preovulatory follicles. In contrast, Zhuang *et al.* (1982) and Welsh *et al.* (1984) reported that clomiphene pretreatment *in vitro* stimulated oestrogen production by rat granulosa cells. In the last two reports, granulosa cells were incubated with clomiphene for 3 days, washed to remove clomiphene, and reincubated with androgen substrate. Oestrogen production was measured after incubation with the androgen substrate, in the absence of clomiphene, and so the 'chronic' effects of clomiphene pretreatment on oestrogen biosynthesis were assessed. During such a long-term exposure,

there may be opportunities for clomiphene to induce changes in cell metabolism, e.g. induction of steroidogenic enzymes.

In the present study, the 'acute' effects of clomiphene on steroidogenesis were assessed. Oestrogen production was inhibited during a 4-h incubation in the presence of clomiphene. Furthermore, washing the cells after a 4-h exposure to clomiphene reversed the inhibitory effects of clomiphene on steroidogenesis, apparently indicating that this brief exposure to clomiphene did not have a permanent effect on cell metabolism. The differences in experimental protocol between the studies above and the present study may account for the different effects of clomiphene on oestrogen biosynthesis. Furthermore, we cannot rule out that species differences may account for the different effects of clomiphene on oestrogen biosynthesis in the studies discussed above and in the present study.

There are also conflicting reports concerning the effects of clomiphene treatment on oestrogen production *in vivo*. Marut & Hodgen (1982) reported that plasma oestrogens decreased during high-dose clomiphene treatment in normally cyclic monkeys despite concurrent elevations of gonadotrophins. Decreased oestrogen concentrations in follicular fluid have also been reported in clomiphene-treated as compared to hMG-treated women (diZerega *et al.*, 1983; Dlugi *et al.*, 1985). However, other reports have described an increase in oestrogen values during clomiphene treatment in monkeys (Littman & Hodgen, 1985) and women (Wu, 1977; Maxson *et al.*, 1984).

Clearly, in some circumstances clomiphene can exert inhibitory actions on ovarian steroidogenesis. Inhibitory effects of clomiphene *in vitro* on progesterone biosynthesis have been reported for rat granulosa cells (Welsh *et al.*, 1984), monkey luteal cells (Westfahl & Resko, 1983), and rat preovulatory follicles (Laufer *et al.*, 1982). In addition to the inhibitory effects reported here on the aromatase and C17–20-lyase activities, clomiphene has been found to inhibit progesterone biosynthesis by an effect on the cholesterol side-chain cleavage enzyme, but not on the 3 β -steroid dehydrogenase enzyme activity, in hen granulosa cells (Sgarlata *et al.*, 1984). Furthermore, an inhibitory effect of clomiphene on cholesterol side-chain cleavage activity was obtained using a mitochondrial preparation of hen granulosa cells (Asem & Hertelendy, 1985), demonstrating a direct effect of clomiphene on this enzyme. It remains to be determined whether the inhibitory effects of clomiphene on the C17–20-lyase and aromatase enzymes in hen theca cells are mediated by a direct effect of clomiphene on the enzymic reactions. However, since the C17–20-lyase, aromatase, and cholesterol side-chain cleavage enzyme activities are all inhibited by clomiphene, this suggests that clomiphene may act via the same mechanism for each enzyme. The cholesterol side-chain cleavage enzyme, as well as the C17–20-lyase and aromatase enzymes, have in common a cytochrome P-450 as part of their system (Lieberman *et al.*, 1984). The cytochrome P-450 or other similarities in these enzyme systems may be the site of action of clomiphene citrate.

In various mammalian systems, clomiphene has been found to exert both antioestrogenic and oestrogenic effects (see Adashi, 1984). The effect of oestradiol-17 β on steroidogenesis by hen theca cells has not been examined and, in the present study, no comparisons were made between the actions of clomiphene and oestradiol-17 β on androstenedione and oestrogen production. We are therefore unable to comment on whether or not the effects of clomiphene that we observed are a result of oestrogenic properties of clomiphene in hen theca cells. However, another study has shown that oestradiol-17 β and clomiphene have different sites of action on the pathway from cholesterol to progesterone in hen granulosa cells (Sgarlata *et al.*, 1984).

Although we acknowledge that there is a problem in extrapolating in-vitro data from hen ovarian cells to the in-vivo human situation, our results confirm other in-vitro studies done on mammalian tissues which indicate that clomiphene has a direct effect on ovarian steroidogenesis. The present study therefore lends support to the ovary as a secondary site of action in the complex mechanism by which clomiphene induces ovulation in women.

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