Effects of enclomiphene and zuclomiphene on basal and gonadotrophin-stimulated progesterone secretion by isolated subpopulations of small and large ovine luteal cells

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We examined the effects of enclomiphene and zuclomiphene, alone and in combination with oestradiol, on basal and gonadotrophin-stimulated progesterone secretion by isolated subpopulations of both large (granulosa-lutein) and small (theca-lutein) ovine luteal cells. Isolated large and small luteal cells derived from intact, enucleated ovine corpora lutea were incubated for 48–120 h with or without 22R-hydroxysterol or pregnenolone (2.5 µM) and a range of enclomiphene, zuclomiphene, and/or oestradiol concentrations (3–100 µM), both with and without ovine luteinizing hormone (100 ng/ml). Spent media were assayed in duplicate for progesterone content by radioimmunoassay. Enclomiphene, zuclomiphene, and oestradiol exhibited equivalent dose-dependent inhibitory effects on basal and gonadotrophin-stimulated small and large ovine luteal cell progesterone secretion under all substrate conditions. Both cell types became more sensitive to clomiphene inhibition with increasing time in culture. In combined treatments, the effects of oestradiol and either enclomiphene or zuclomiphene became additive in longer-term cultures and were never antagonistic. In this model system, (i) clomiphene, like oestradiol, appears to inhibit 3β-hydroxysteroid dehydrogenase activity, (ii) both stereoisomers act as oestrogen agonists, (iii) neither demonstrates any anti-oestrogenic properties, and (iv) both large and small luteal cells become more sensitive to clomiphene inhibition with increasing duration of exposure.

Key words: clomiphene citrate/in vitro/lutein cells/ovine

Introduction

In-vitro studies conducted with luteinized granulosa cells (Sgarlata et al., 1984; Yuen et al., 1988) and luteal tissue (Hammerstein, 1969) suggest that clomiphene can directly inhibit luteal cell steroidogenesis by interfering with cholesterol uptake and/or side chain cleavage activity (Hammerstein, 1969; Sgarlata et al., 1984; Yuen et al., 1988). Oestradiol, by contrast, exerts similar inhibitory effects at a more distal site, on 3β-hydroxysteroid dehydrogenase (3β-HSD) (Sgarlata et al., 1984). In luteinized avian granulosa cells, triphenylethylene anti-oestrogens inhibit gonadotrophin-stimulated steroidogenesis (Sgarlata et al., 1984), whereas in human cells (Yuen et al., 1988; Hammerstein, 1969) clomiphene citrate inhibits only basal progesterone secretion and the effect is blocked by treatment with human chorionic gonadotrophin (HCG). Because they secrete large amounts of progesterone but virtually no oestrogens, luteinized avian granulosa cells have advantages over human cells derived from in-vitro fertilization cycles for investigations of anti-oestrogen actions. However, neither model system represents true luteal cells, and previous studies in either system have used only racemic clomiphene citrate; the specific effects of enclomiphene and zuclomiphene, two distinct stereoisomers, have not been investigated.

In this study, we examined the effects of varying concentrations of enclomiphene and zuclomiphene on basal gonadotrophin-stimulated progesterone production by cultured ovine luteal cells which, like the avian model (Sgarlata et al., 1984), secrete virtually no oestrogen. In contrast to previous studies with luteinized granulosa cells (Sgarlata et al., 1984; Yuen et al., 1988), we conducted our investigations with true luteal cells derived from intact, enucleated ovine corpora lutea. To clarify the influence of gonadotrophins, we performed parallel experiments in isolated subpopulations of both 'large' (granulosa-lutein) and 'small' (theca-lutein) luteal cells; in this model system, only small luteal cells respond to gonadotrophin stimulation (Fritz et al., 1991). To investigate further the site where clomiphene and oestradiol inhibit steroidogenesis in vitro, we examined the effects of both, alone and in combination, in experiments wherein 22R-hydroxycholesterol (a naturally occurring cholesterol side-chain cleavage intermediate) or pregnenolone (the immediate precursor for 3β-HSD) was provided as steroidogenic substrate.

Materials and methods

Materials

The cholesterol analogue 5-cholesten-3β,22(R)-diol (22R-OHC) and oestradiol were obtained from Sigma (St Louis, MO, USA). Ovine...
luteinizing hormone (oLH) was a gift from the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases. Enclomiphene and zuclohimcne were donated by Merrell Dow Pharmaceuticals (Cincinnati, OH, USA). Culture medium was purchased from Whittaker MA Bioproducts (Walkersville, MD, USA), fetal bovine serum from Hy-Clone (Logan, UT, USA), and culture dishes and pipettes from Costar (Cambridge, MA, USA). Insulin/transferrin/selenium premix (ITS) was obtained from Collaborative Research (Bedford, MA, USA). Antiserum for progesterone (#337) was a gift from Dr G.D. Niswender (Fort Collins, CO, USA).

**Tissue processing**

The study was approved by the local institutional review board and guidelines for the care and use of animals were followed throughout. Ovine corpora lutea were obtained from superovulated ewes on days 8–10 of the oestrous cycle (day 0 = oestrus), enzymatically dispersed, elutriated, and cryopreserved as previously described (Fitz et al., 1982; Balapure et al., 1989). Briefly, each batch of cells was derived from a group of three to four cyclic ewes which were ovarioctomized after superovulation by treatment with porcine follicle stimulating hormone. Corpora lutea were enucleated intact, dissected free from connective tissues, serially sectioned with a hand-held Stadie-Riggs microtome, and dissociated with collagenase in a shaking water bath at 37°C under O2. Dissociated cells were elutriated to yield highly enriched subpopulations of large (diameter >22 mm) and small (diameter <22 mm) steroidogenic cells. Small cells preparations were essentially devoid of large cells whereas large cell fractions had modest but significant small cell contamination (2.98 ± 0.48:1 large:small cells; n = 8). Cell fractions were suspended in ice-cold medium containing 7.5% dimethylsulphoxide, frozen in a two-step temperature gradient, and stored at -196°C until thawed immediately before use. Incubations were performed with aliquots of cells recovered after 10–90 days of cryostorage. Under these conditions, ovine luteal cells remain viable and retain normal morphological and functional characteristics (Balapure et al., 1989; Fitz et al., 1993). Cell morphology, viability, and function were assessed in representative fractions by microscopy, Trypan Blue exclusion, and expression of 3β-HSD activity, as previously described (Balapure et al., 1989). There were no changes in any of these parameters throughout the culture period.

**Cell incubations**

Luteal cells were distributed among tissue culture dishes (approximately 10^6 large cells or 4×10^4 small cells/22 mm well) and allowed to attach by overnight incubation in medium (M199 containing 0.1% bovine serum albumin (BSA), 20 mM HEPES, 5 μg/ml transferrin, 5 ng/ml selenium, 50 U/ml penicillin and 50 μg/ml streptomycin, pH 7.35) containing 2% fetal bovine serum under 5% CO2 at 37°C. Plating medium was removed and cells were washed twice with serum-free medium before treatment. At frequent intervals throughout each incubation, we inspected representative wells and observed no evidence of significant cell detachment or death under any treatment conditions.

Steroids were dissolved in absolute ethanol before dilution in culture medium; enclomiphene and zuclohimcne were dissolved first in dimethylsulphoxide, then serially diluted in ethanol before addition to medium. Within each experiment, the final medium concentration of ethanol was constant and always <1%, a level which had no detectable effects on cell viability or function in preliminary studies. Each experiment was performed four times, on each occasion with a different batch of luteal cells. Within each experiment, treatment conditions were conducted in triplicate with 1.0 ml medium/well.

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Media were harvested and replaced with fresh media of identical composition at 6, 24, 48 and 120 h.

**Experimental design**

Elutriated subpopulations of small and large ovine luteal cells were incubated in plain medium or medium supplemented with 22R-OHC (2.5 μM) or pregnenolone (2.5 μM). All treatment conditions were repeated with addition of a maximum-stimulating concentration of oLH (100 ng/ml). In the first experiment, enclomiphene and zuclohimcne were added over a range of concentrations (0, 3, 10, 33, 100 μM). In the second experiment, we investigated the effects of combined treatment with oestradiol and the clomiphene isomers, each at two different concentrations (5 and 25 μM). Each experiment was repeated four times and within each experiment all conditions were performed in triplicate.

**Progesterone radioimmunoassay**

Spent media from luteal cell incubations were frozen immediately after harvest and stored at -20°C until assayed in duplicate for progesterone content by radioimmunoassay as previously described (Fitz et al., 1993). Crossreactivity of 22R-OHC and pregnenolone in this assay was <0.01% and intra- and interassay coefficients of variation were <10%. Representative aliquots of non-incubated treatment medium were reserved after each experiment and invariably contained no detectable progesterone.

**Statistical analysis**

To control for variations in the performance of different cell batches and to standardize the data for statistical analysis, progesterone secretion by treated cells (substrate supplementation, oLH, clomiphene and/or oestradiol) was expressed in relation to the amount produced under basal conditions (no treatment) in each experiment of each replicate (defined as 100%); data (means ± SEM) from all four repeated experiments were then combined. Data in Figure 1 are expressed in absolute terms, for reference. Treatment effects were compared by multivariate analysis of variance and when different (P < 0.05), individual means were compared by the Bonferroni multiple range test. Computerized statistical analyses were performed with the Systat statistical program (Systat, Inc., Evanston, IL, USA).

**Results**

The influence of substrate supplementation with 22R-OHC or pregnenolone on absolute values of basal and oLH-stimulated small and large luteal cell progesterone secretion are shown in Figure 1. Consistent with our previous observations (Fitz et al., 1982) in the absence of substrates, basal large cell progesterone production was three to 10 times greater than in small cells but small cells responded more dramatically to gonadotrophin stimulation. The modest oLH-induced increase in large cell progesterone secretion seen in longer-term incubations was consistent with the extent of small cell contamination in these preparations. As expected, both cell types maintained higher values of progesterone secretion when provided with a ready source of steroidogenic substrate (P < 0.05). Pregnenolone was more efficiently metabolized than 22R-OHC (P < 0.05) and oLH had no demonstrable tropic effects in the presence of either substrate (Figure 1).

Representative data shown in Figures 2–5 are limited only by space constraints; cell type (small or large) and conditions (time in culture, ± oLH and/or 22R-OHC, pregnenolone) are
The effects of clomiphene and its two stereoisomers on basal and gonadotrophin-stimulated ovine luteal cell progesterone secretion were investigated. Clomiphene and its isomers were added to cultures of ovine luteal cells in vitro, and the effects on progesterone secretion were measured under basal and gonadotrophin-stimulated conditions. The results demonstrated that clomiphene directly inhibits ovine luteal cell progesterone secretion in vitro. Similar observations have been made in other luteal tissues, and the unique features of our model system contrast with previous investigations. In short-term cultures (6 h), like enclomiphene and zuclomiphene, at similar concentrations (5, 25 μM), oestradiol clearly inhibited luteal cell progesterone secretion in a dose-dependent manner (P < 0.05); combined treatment with enclomiphene or zuclomiphene (5, 25 μM) had no additional or antagonistic effects. However, after longer durations in incubation (48 h), the inhibitory effects of oestradiol and enclomiphene became additive (P < 0.05); at either concentration of oestradiol (5, 25 μM), addition of enclomiphene or zuclomiphene (5, 25 μM) resulted in a stepwise decrease in progesterone secretion (P < 0.05).

Effects of enclomiphene and zuclomiphene on progesterone production by unstimulated small luteal cells incubated (48 h) in the presence of 22R-OHC or pregnenolone and enclomiphene are shown in Figure 5. The two stereoisomers exhibited equivalent dose-dependent inhibitory effects in the presence of both substrates (P < 0.05).

Discussion
The results of our study demonstrate that clomiphene directly inhibits ovine luteal cell progesterone secretion in vitro. Similar observations have been made in other luteal tissues (Hamerstein, 1969; Sgarlata et al., 1984; Olsson et al., 1987; Yuen et al., 1988) but the unique features of our model system and experimental design address questions left unanswered in previous investigations. Specifically, (i) we conducted our experiments with true luteal cells derived from intact corpora lutea as opposed to luteinized granulosa cells, (ii) we performed parallel incubations in isolated subpopulations of both 'large' (granulosa–lutein) and 'small' (theca–lutein) luteal cells, and (iii) we examined the effects of enclomiphene and zuclomiphene individually rather than in racemic mixture, both alone and in combination with oestradiol, all in a system which avoids the confounding effects of endogenous oestrogen production.

In systems classically used to assess oestrogenic potency, clomiphene and clomiphene exhibited mixed agonistic and antagonistic properties; both effects can be demonstrated across a range of concentrations (Clark et al., 1982). Although the relative oestrogenic and anti-oestrogenic effects of enclomiphene and zuclomiphene may vary widely, even within the same tissue (e.g. endometrial epithelial versus stromal cells) (Clark and Markaverich, 1982), the two stereoisomers had strikingly similar inhibitory effects on small and large ovine luteal cell progesterone secretion. Dose–response curves for enclomiphene and zuclomiphene were virtually identical and similar to that for oestradiol. Moreover, when combined, the effects of oestradiol and either enclomiphene or zuclomiphene became additive with time in culture and were never antagonistic. Thus, in this model system, both clomiphene and isomers acted as oestrogen agonists and neither exhibited any anti-oestrogenic effects.

In luteinized avian granulosa cells (Sgarlata et al., 1984), clomiphene clearly inhibits oLH-stimulated progesterone secretion, but in some human luteal tissues (Hamerstein, 1969; Yuen et al., 1988) its effects are blocked by HCG. In our
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Figure 2. Effects of varying concentrations (as shown) of enclomiphene (En) and zuclomiphene (Zu) on basal (open bars) and ovine luteinizing hormone (oLH)-stimulated (hatched bars) progesterone secretion by small luteal cells after 6 h incubation. See text for details. Progesterone concentrations are expressed in relation to that amount produced by untreated cells (defined as 100%). Data are means ± SEM and represent the combined results of four experiments. Both stereoisomers inhibited basal and gonadotrophin-stimulated luteal cell progesterone secretion in a dose-dependent manner and to an equivalent degree (P < 0.05). CC = clomiphene citrate.

Figure 3. Effects of 10 μM enclomiphene (open bars) and zuclomiphene (hatched bars) on progesterone secretion by unstimulated small luteal cells after increasing durations of incubation (as shown). Cells were cultured in media supplemented with 22R-hydroxycholesterol (22R-OHC), pregnenolone, or in the absence of substrates as indicated. See text for details. Progesterone concentrations are expressed in relation to that amount produced under basal conditions at each point in time (defined as 100%). Data are means ± SEM and represent the combined results of four experiments. Clomiphene (10 μM) inhibited progesterone production to a progressively greater extent with the passage of time (P < 0.001), although the effect was least dramatic in the presence of pregnenolone (P < 0.02). The effects of enclomiphene and zuclomiphene were statistically indistinguishable.

studies, conducted with highly enriched subpopulations of both large (high basal secretion, gonadotrophin insensitive) and small (low basal secretion, gonadotrophin sensitive) ovine luteal cells, enclomiphene and zuclomiphene inhibited both basal and oLH-stimulated progesterone production. Although differences in species, experimental design, and choice of gonadotrophins might well explain the conflicting results of earlier studies, on close examination, our data are consistent with those obtained in both avian (Sgarlata et al., 1984) and human (Olsson et al., 1987) luteal tissues. Overall, gonadotrophin treatment had no influence on the effects of clomiphene in either large or small luteal cells. While it is possible that HCG might block clomiphene's inhibitory effects more effectively than oLH, we chose the more species-specific gonadotrophin for our studies.

Previous studies have suggested that clomiphene inhibits luteal cell progesterone production by interfering with cholesterol uptake and/or side-chain cleavage activity (Hammerstein, 1969; Sgarlata et al., 1984; Olsson et al., 1987; Yuen et al., 1988) whereas oestradiol exerts similar effects on 3β-HSD (Sgarlata et al., 1984). In luteinized avian granulosa cells, clomiphene effectively blocked metabolism of 25-hydroxycholesterol but had no effect on conversion of pregnenolone to progesterone; in contrast, oestradiol arrested metabolism of the cholesterol analogue at pregnenolone and blocked conversion of this more immediate precursor (Sgarlata et al., 1984).
Figure 4. Effects of two concentrations (5, 25 μM) of oestradiol (E2), enclomiphene (open bars), and zuclomiphene (hatched bars), alone and in combinations, on unstimulated large luteal cells after 6 h (top panel) and 48 h (lower panel) incubation in the presence of pregnenolone (2.5 μM). See text for details. Progesterone concentrations are expressed in relation to that amount produced by unsupplemented cells under basal conditions (defined as 100%). Data are means ± SEM and represent the combined results of four experiments. Values denoted by different letters are different from each other (P < 0.05) and from control (no treatment). CC = either enclomiphene or zuclomiphene, as appropriate.

Figure 5. Effect of varying concentrations (as shown) of enclomiphene (open bars) and zuclomiphene (hatched bars) on progesterone production by unstimulated small luteal cells after 48 h incubation in the presence of 22R-hydroxycholesterol (22R-OHC) or pregnenolone. See text for details. Progesterone concentrations are expressed in relation to that amount produced by untreated cells under basal conditions (defined as 100%). Data are means ± SEM and represent the combined result of four experiments. Values denoted by one or two asterisks are different (P < 0.05) from control (no treatment) and each other.

1984). In our studies, both enclomiphene and zuclomiphene consistently inhibited progesterone secretion regardless of whether media were supplemented with 22R-OHC or pregnenolone. Oestradiol was an equally if not more effective inhibitor under all conditions. Our data are consistent with previous indications that oestradiol inhibits 3β-HSD activity, but also suggest that clomiphene inhibits 3β-HSD in ovine luteal cells.

Our use of 22R-OHC and pregnenolone as steroidogenic substrates was intended as a means to differentiate between inhibition of side-chain cleavage and 3β-HSD. Inhibition at the more distal site was unexpected and effectively obscured any similar actions clomiphene may have on cholesterol side-chain cleavage.

In considering the clinical implications of these data, the concentration at which clomiphene inhibits luteal cell progesterone secretion deserves careful analysis. Many contend that plasma clomiphene concentrations never reach toxic levels (Mishell, 1986). However, pharmacokinetic studies suggest that systemic accumulation of at least one isomer (zuclomiphene) is likely after repeated cycles of treatment (Mikkelsen et al., 1986; Fritz et al., 1991); the concentrations that ultimately may be achieved have therefore not been well defined. In our model system, the concentrations required to inhibit
progesterone synthesis were similar to those observed in studies with luteinized avian granulosa cells (IC\textsubscript{50}: 3 \textmu M) (Sgarlata et al., 1984), but evidence indicates that human luteal cells are sensitive to significantly lower levels (300 nM), even in short-term incubations (Hammerstein, 1969; Yuen et al., 1988). Our data suggest that the concentrations required to inhibit progesterone production may be lower still after longer durations of exposure in vivo. Moreover, the added inhibitory effects of oestradiol may further reduce the concentration of clomiphene required to inhibit corpus luteum progesterone secretion. Observations that luteal phase progesterone concentrations are often higher, rather than lower, after clomiphene treatment than in spontaneous cycles clearly question the clinical significance of the inhibitory effects observed in vivo in this and in previous studies (Hammerstein, 1969; Sgarlata et al., 1984; Yuen et al., 1988). However, these higher steroid concentrations result from multiple ovulations (Yeko et al., 1990; Guzick and Zeleznik, 1990) and thus represent the combined contributions of more than a single corpus luteum. They indicate that any adverse effects of clomiphene are often obscured or effectively overcome by the increase in luteal cell mass that accompanies clomiphene multiple ovulation, but do not rule out clinically significant inhibition of luteal cell steroid biosynthesis altogether.

In summary, enclomiphene and zuclopenthixol exhibit equivalent, dose-dependent inhibitory effects on basal and gonadotrophin-stimulated small and large ovine luteal cell progesterone secretion in vitro. In this model system, clomiphene appears to inhibit 3\beta-HSD activity, both stereoisomers act as oestrogen agonists, neither demonstrates any anti-oestrogenic properties, and sensitivity to clomiphene inhibition increases with duration of exposure.

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**References**


