Changes in oestrogen biosynthesis in preovulatory rat follicles after blockage of ovulation with pentobarbitone sodium

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Summary. Follicles isolated 1 and 2 days after pentobarbitone sodium injection at pro-oestrus were incubated with C-21 steroids or aromatizable C-19 steroids. Addition of testosterone or androstenedione (50 ng/ml) increased oestradiol production by ovulation-blocked follicles, while addition of progesterone or 17α-hydroxyprogesterone was ineffective. LH-stimulated oestradiol production was lower in follicles isolated 1 and 2 days after pentobarbitone sodium injection, but progesterone production was elevated compared to pro-oestrous follicles. Total steroidogenesis, measured by pregnenolone production in the presence of inhibitors of pregnenolone conversion, did not differ on the 3 days. The activity of C17–20 lyase, measured in follicular homogenates, decreased between pro-oestrus and the next day. Aromatase and 17α-hydroxylase activities also decreased, but the activity of these enzymes was always considerably higher than that of C17–20 lyase.

It is concluded that the decrease in follicular oestradiol production after injection of pentobarbitone sodium was due primarily to a decrease in the activity of the enzyme system responsible for the conversion of 17α-hydroxyprogesterone to androstenedione, thereby limiting the amount of substrate available for aromatization to oestrogen.

Introduction

Blockage of ovulation in the rat by injection of pentobarbitone sodium early in the afternoon of the day of pro-oestrus and the next day results in atresia of preovulatory follicles (van der Schoot, 1978). This procedure has been used to study temporal changes of preovulatory follicles becoming atretic in rats (Uilenbroek, Woutersen & van der Schoot, 1980; Braw & Tsafriri, 1980) and hamsters (Terranova, 1981). By 1 and 2 days after pro-oestrus uniovulated follicles are histologically not different from pro-oestrous follicles. The first morphological signs of atresia (i.e. pycnosis of granulosa cells) appeared 3 days after pro-oestrus (Uilenbroek et al., 1980). The biochemical changes so far studied revealed a decrease in follicular oestradiol production between pro-oestrus and 1 day after pro-oestrus. Androgen production was also decreased, but progesterone production was stimulated (Braw & Tsafriri, 1980). It was therefore, suggested that the diminished production of oestradiol was due to an impaired conversion of progesterone to aromatizable androgens.

In the present study follicles isolated at pro-oestrus and 1 and 2 days after pentobarbitone injection were incubated in the presence of LH to test the ability of these follicles to produce oestradiol and progesterone in vitro. To investigate whether the low follicular oestradiol production after pentobarbitone was due to a decreased conversion of progesterone to androgens, follicular oestradiol production was measured after addition of various substrates, and the activity of
enzymes responsible for the conversion of progesterone to oestradiol was measured in follicular homogenates. Preliminary results have been published by Uilenbroek, van der Schoot, den Besten & Woutersen (1982).

**Materials and Methods**

*Animals.* Adult female rats of Wistar origin were used. The animals were 3–5 months old and housed under controlled conditions (20–23°C, lights on 05:00–19:00 h). The animals showed predominantly 5-day reproductive cycles as assessed from daily vaginal smears. Only animals showing 2 consecutive 5-day cycles were used. Experimental animals were given a single i.p. injection of pentobarbitone sodium (37 mg/kg body weight) between 13:00 and 13:30 h on the day of pro-oestrus to prevent ovulation. To prevent delayed ovulation a second injection was given the next day between 13:30 and 14:00 h. At pro-oestrus, Day 1 (1 day after injection of pentobarbitone at pro-oestrus) and at Day 2 (1 day after injection of pentobarbitone at pro-oestrus and the next day) animals were killed between 09:00 and 11:00 h. The ovaries were excised and the 10–12 largest follicles were isolated. These follicles were assumed to be those that would have ovulated, had the pentobarbitone treatment not been given. This assumption seemed justified as it appeared that the number of ova collected in the oviduct after ovulation corresponded with the number of large follicles.

*Follicle incubations.* Individual follicles were incubated in 500 µl Medium 199 (Gibco, Grand Island, NY) pH 7·4 containing 10 mM HEPES (Sigma, St Louis, MO). The incubations were carried out at 37°C under an atmosphere of 95% O₂ and 5% CO₂ in a shaking waterbath. After incubation for 4 h the follicles were discarded and the medium was stored at −20°C until assay for steroids.

In Exp. 1 follicles isolated at pro-oestrus, Day 1 and Day 2 were incubated in the absence and presence of LH (NIH-LH-S19, LH activity 1·01 x NIH-LH-S1, FSH activity 0·05 x NIH-FSH-S1) in a dose ranging from 1 to 100 ng/ml. After incubation for 4 h oestradiol and progesterone concentrations in the medium were measured. To measure total steroid production follicles were incubated in medium which contained, in addition to LH, an inhibitor of 3β-hydroxysteroid dehydrogenase activity (cyanoketone, 2α-cyano-4,4′,17α-trimethyl-17β-hydroxy-5-androsten-3-one: Sterling-Winthrop, New York; 2 µg/ml) and an inhibitor of 17α-hydroxylase activity (SU-10603, 7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(2H)-naphthalenone: Ciba-Geigy, Basel, Switzerland; 5 µg/ml). The optimal concentrations of inhibitors of pregnenolone metabolism were chosen on the basis of the results of studies by van Beurden, Roodnat & van der Molen (1978). Accumulation of pregnenolone in the medium was measured after incubation for 4 h. To detect the effectiveness of these inhibitors follicles from rats at pro-oestrus were incubated with LH (20 ng/ml) in the absence and presence of cyanoketone and SU-10603. In the absence of inhibitors, oestradiol production was 9470 ± 690 pg/follicle/2 h, while in the presence of inhibitors only 32 ± 3 pg/follicle/2 h was found (n = 10). Furthermore, incubation in the presence of 3H-labelled pregnenolone revealed, after extraction and thin layer chromatography (t.l.c.), one peak of radioactivity. From the total amount recovered, 86% was pregnenolone, identified by recrystallization to constant specific activity.

In Exp. 2 follicles isolated at pro-oestrus, Day 1 and Day 2 were incubated in medium with and without addition of substrate: testosterone, androstenedione, 17α-hydroxyprogesterone or progesterone at a final concentration of 50 ng/ml. After incubation for 4 h oestradiol in the medium was measured.

*Steroid assays.* The accumulation of oestradiol-17β and progesterone in the medium was measured by radioimmunoassay without prior extraction as described previously (Uilenbroek et al., 1980). The sensitivity of the oestradiol assay was 10 pg/tube and the coefficient of variation between assays was 8.3%. The sensitivity of the progesterone assay was 25 pg/tube and the coefficient of variation between assays was 16%. Addition of testosterone, androstenedione, 17α-
hydroxyprogesterone or progesterone to the culture medium (50 ng/ml) did not interfere with the oestradiol assay. The concentration of pregnenolone in the medium was measured by radioimmunoassay as described by van der Vusse, Kalkman & van der Molen (1975). The sensitivity of the pregnenolone assay was 25 pg/tube and the coefficient of variation between assays was 2.5%.

**Enzyme activity.** Pools of 10 follicles, each pool isolated from ovaries of a single rat, were homogenized in 1 ml 0-05 m-Tris/EDTA-buffered saline pH 7-4 and centrifuged at 800 g (20 min, 4°C). Samples (250 µl) of the supernatant were incubated with 3H-labelled substrate (5 × 10^5 d.p.m.), 1 µg unlabelled substrate, 500 µl of a NADPH-generating system (containing 1:4 U glucose 6-phosphate dehydrogenase, 20 mm-glucose 6-phosphate and 2 µM-NADP) and 250 µl of an appropriate inhibitor. For determination of aromatase activity, 4-androsten-3-one-17β carboxylic acid (AC, 126 µg/ml) was used to inhibit 5α-reductase activity. For determination of 5α-reductase activity, androsta-1,4,6-triene-3,17-dione (ATD, 113 µg/ml) was added to inhibit aromatase activity, while for the measurement of 17α-hydroxylase and C17–20 lyase activity both AC and ATD were added. [7(n)-3H]Testosterone (NEN, Boston, MA; sp. act. 25 Ci/mmole plus 1 µg (3-5 nmol) testosterone were used as substrate for the determination of aromatase and 5α-reductase activity. [1,2,6,7-3H]Progesterone (Radiochemical Centre, Amersham, U.K.; sp. act. 93 Ci/mmole) plus 1 µg (3-2 nmol) progesterone and [1,2,6,7-3H]17α-hydroxyprogesterone (Radiochemical Centre; sp. act. 63 Ci/mmole) plus 1 µg (3-0 nmol) 17α-hydroxyprogesterone were used as substrates for the measurement of 17α-hydroxylase and C17–20 lyase activity respectively. The incubations were started by adding 250 µl supernatant of the follicle homogenate and were carried out for 30 min at 37°C in a shaking waterbath in an atmosphere of 95% O2 and 5% CO2. The metabolic products formed were extracted, separated by t.l.c. and counted. The metabolic products were testosterone, 5α-dihydrotestosterone and 5α-androstan-3α,17β-diol for 5α-reductase; progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone for 17α-hydroxylase; 17α-hydroxyprogesterone, androstenedione and testosterone for C17–20 lyase; and testosterone, oestradiol-17β, oestrone and oestriol for aromatase. The enzyme activity was expressed as pg substrate converted per follicle per min.

**Statistical analysis.** The results have been expressed as means ± s.e.m. Statistical analysis consisted of two-way analysis of variance. Provided that the overall test was significant, comparisons between groups were made by Duncan’s multiple range test. A probability of 0.05 or less was considered to be statistically significant.

**Results**

**Follicle incubations**

Accumulation of oestradiol-17β in the medium after incubation for 4 h decreased from 2163 ± 155 pg/follicle/4 h by follicles isolated at pro-oestrus to 516 ± 77 and 264 ± 73 pg/follicle/4 h by follicles isolated at Day 1 and Day 2 respectively (Text-fig. 1a). Addition of LH to the culture medium increased oestadiol production in a dose-dependent manner by follicles isolated at pro-oestrus. A significantly lower dose–response curve was found for Day 1 follicles. For Day 2 follicles, LH-stimulated oestadiol production was further decreased. In contrast to oestadiol, basal and LH-stimulated progesterone production were higher for Day 1 and Day 2 follicles than for those at pro-oestrus (Text-fig. 1b). LH-stimulated progesterone production was not different between Days 1 and 2. Accumulation of pregnenolone (pg/follicle/4 h) in LH-free medium, in which further conversion of pregnenolone was inhibited, was significantly lower at Day 2 (859 ± 173) than at pro-oestrus (2540 ± 379) and Day 1 (2161 ± 258) (Text-fig. 1c). However, LH-stimulated pregnenolone production was not different between the 3 days studied. With higher doses of LH, pregnenolone production was lower than that of progesterone.
Text-fig. 1. Accumulation of (a) oestradiol, (b) progesterone and (c) pregnenolone in the medium by individual follicles in the absence (C) or presence of LH. Follicles were isolated at pro-oestrus, 1 day after injection of pentobarbitone at pro-oestrus (Day 1) and 1 day after injection of pentobarbitone at pro-oestrus and Day 1 (Day 2). In (c) the medium contained an inhibitor of 3β-hydroxysteroid dehydrogenase activity (cyanoketone, 2 µg/ml) and an inhibitor of 17α-hydroxylase activity (SU-10603, 5 µg/ml). Each point represents the mean ± s.e.m. of 8–10 follicles (2 follicles per rat).
Text-fig. 2. Accumulation of oestradiol-17β in the medium by individual follicles in the absence (C) or presence of various substrates (50 ng/ml). Follicles were isolated at pro-oestrus, 1 day after injection of pentobarbitone at pro-oestrus (Day 1) and 1 day after injection of pentobarbitone at pro-oestrus and Day 1 (Day 2). Each value represents the mean ± s.e.m. of 8–10 follicles (2 follicles per rat). *P < 0.001 compared with control group (C).

Oestradiol production after addition of aromatizable androgens (testosterone or androstenedione) to the medium was significantly increased by follicles isolated at Day 1 and Day 2 (Text-fig. 2). No such increase was found after addition of progesterone or 17α-hydroxyprogesterone.

Enzyme activity

The activities of 17α-hydroxylase, C17-20 lyase, aromatase and 5α-reductase measured in homogenates of follicles isolated at pro-oestrus, Day 1 and Day 2 are given in Table 1. Between pro-oestrus and Day 1 the activity of 17α-hydroxylase and C17–20 lyase decreased significantly. A decrease of aromatase activity and a further decrease of C17–20 lyase activity was found between Day 1 and Day 2. At all days studied the activity of C17–20 lyase was significantly lower than that of 17α-hydroxylase or aromatase activity (P < 0.001). The activity of 5α-reductase in pro-oestrous follicles was low and did not change after pentobarbitone treatment.

Table 1. Activity of various enzymes in the 10–12 largest follicles isolated at pro-oestrus, 1 day after injection of pentobarbitone sodium at pro-oestrus (Day 1) and 1 day after injection of pentobarbitone sodium at pro-oestrus and the next day (Day 2)

<table>
<thead>
<tr>
<th>Large follicles isolated at</th>
<th>17α-Hydroxylase (pg/follicle/min)</th>
<th>C17-20 lyase (pg/follicle/min)</th>
<th>Aromatase (pg/follicle/min)</th>
<th>5α-Reductase (pg/follicle/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pro-oestrus</td>
<td>1156 ± 90</td>
<td>435 ± 33</td>
<td>3868 ± 248</td>
<td>393 ± 30</td>
</tr>
<tr>
<td>Day 1</td>
<td>514 ± 75*</td>
<td>134 ± 18†</td>
<td>2802 ± 388</td>
<td>397 ± 94†</td>
</tr>
<tr>
<td>Day 2</td>
<td>532 ± 72*</td>
<td>57 ± 16††</td>
<td>829 ± 90††</td>
<td>456 ± 51††</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. of 5–6 animals.
* P < 0.01, †P < 0.001 compared with value at pro-oestrus.
‡P < 0.001 compared with value at Day 1.
Discussion

The present results confirm and extend previous observations by showing that blockade of ovulation in rats by an injection of pentobarbitone at pro-oestrus results in a rapid decrease in follicular oestradiol production (Uilenbroek et al., 1980; Braw & Tsafriri, 1980; Terranova, 1981). From these studies it was suggested that this decrease in oestradiol production was due to a decreased ability of unovulated follicles to convert progesterone to androstenedione, so limiting the amount of substrate available for aromatization. The present results substantiate this suggestion by showing that addition of aromatizable androgens (testosterone and androstenedione) to the culture medium increased oestradiol production in vitro by intact unovulated follicles, while addition of progesterone or 17α-hydroxyprogesterone was ineffective. Furthermore, estimations of enzyme activity in follicular homogenates revealed a 70% decrease in C17–20 lyase activity between pro-oestrus and Day 1 and a further decrease 2 days after injection of pentobarbitone. Also, 17α-hydroxylase and aromatase activity decreased between pro-oestrus and Day 2. However, the activity of these enzymes was always much higher than that of C17–20 lyase. This demonstrated that the decrease in follicular oestradiol synthesis after pentobarbitone was primarily due to a decrease in the activity of the enzyme converting C-21 steroids to C-19 steroids.

Addition of LH to the culture medium increased oestradiol and progesterone production in pro-oestrous follicles. In follicles isolated at Day 1 LH-stimulated oestradiol production was significantly lower, while LH-stimulated progesterone production was significantly higher than that in follicles isolated at pro-oestrus. There seems to be a discrepancy in that while addition of progesterone to the culture medium (50 ng/ml) did not increase oestradiol production in Day 1 follicles, addition of 10 ng LH, resulting in a progesterone production of about 50 ng/ml, increased oestradiol 4-fold. However, it has been demonstrated that endogenously produced progesterone is preferentially converted compared to exogenous progesterone (Matsumoto & Samuels, 1969). An alternative explanation might be that LH stimulated enzyme activities distal to cholesterol–pregnenolone conversion. Furthermore, a small increase in oestradiol production by Day 1 follicles is not incompatible with the conclusion reached above that C17–20 lyase activity is decreased after pentobarbitone treatment because some enzyme activity is still present. Also, Toorop & Gribilng-Hegge (1982) reported an increase in the ovarian content of oestradiol during the LH surge on the afternoon of Day 1.

Pregnenolone production in the presence of cyanoketone and SU-10603 was used as an indicator for total steroidogenic activity (van Beurden et al., 1978). Pregnenolone production by follicles taken on pro-oestrus and Day 1 did not differ, indicating that total steroidogenic activity was not decreased after pentobarbitone treatment. A lower pregnenolone production was found in LH-free medium for Day 2 follicles, but LH-stimulated pregnenolone production was not different between the 3 days studied. In agreement, the LH receptor content in granulosa cells did not decrease between pro-oestrus and Day 1 (Uilenbroek et al., 1980). Although cyanoketone and SU-10603 were very effective in blocking oestradiol production after stimulation with 20 ng LH, progesterone production after stimulation with higher doses of LH exceeded that of pregnenolone at Days 1 and 2. Either this enzyme block was incomplete after stimulation with high doses of LH or side-chain cleavage enzymes were also inhibited. A similar observation was made by Rommerts, van Roemburg, Lindh, Hegge & van der Molen (1982) who, for cultured rat Leydig cells, found higher LH-stimulated testosterone than pregnenolone production with the same concentrations of these inhibitors as used in the present study.

Besides a decrease in the production of aromatizable androgens the amount of these androgens might have been further reduced by an increase in 5α-reductase activity. However, the activity of this enzyme in the follicles did not change between pro-oestrus and Day 1 (Table 1). In agreement androsterone accumulation in the culture medium decreased from 980 ± 86 pg/follicle/4 h at pro-oestrus to 381 ± 24 pg/follicle/4 h at Day 1 (J. Th. J. Uilenbroek, unpublished results). An increase in 5α-reductase activity does not therefore seem to be involved in the decrease of follicular oestrogen production after pentobarbitone treatment.
The alteration in oestrogen, androgen and progesterone synthesis after pentobarbitone treatment resembles the oestrogen–progesterone shift late on the afternoon of pro-oestrus after the ovulatory LH surge. Follicles isolated 4–6 h after the onset of the endogenous LH surge produced low amounts of oestradiol, androstenedione and testosterone, while progesterone production was stimulated (Hillensjö, Bauminger & Ahrén, 1976). Cleavage of the 17:20 sidechain rather than the aromatase enzyme limited oestriadiol synthesis in the preovulatory follicles after the gonadotrophin surge (Hillensjö, Hamberger & Ahrén, 1977). In the study by Hillensjö et al. (1977) the controls were pentobarbitone-treated rats in which oestrogen–progesterone shift had not occurred up to 20:00 h on the day of pro-oestrus. The present findings demonstrated that not only in the presence, but also in the absence of a preovulatory LH surge, C17–20 lyase activity becomes decreased although the time course is different.

With regard to the possible mechanism whereby in the absence of a preovulatory LH surge follicular oestriadiol production is decreased, it is unlikely that this is due to a direct effect of pentobarbitone or to a pentobarbitone-induced decrease in the tonic LH secretion because in 4-day cyclic rats and hamsters the decrease in oestriadiol production occurs on the 2nd rather than the 1st day after pentobarbitone treatment (Braw & Tsafriri, 1980; Terranova, 1981). Therefore, in 4-day cyclic rats an additional injection of pentobarbitone has to be given to prevent spontaneous ovulation.

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References


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