Effect of human chorionic gonadotrophin and indomethacin on ovulation, steroidogenesis and prostaglandin synthesis in preovulatory follicles of PMSG-primed immature rats*

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Summary. Immature rats were treated with PMSG followed 56 h later by 10 i.u. hCG. Follicles were removed at intervals after hCG injection. Transient increases in progesterone, testosterone and oestradiol synthesis were first evident 1 h after hCG, but values peaked at 3–5 h and returned to control levels by 10 h. Increased synthesis of PGE-2 and PGF-2α was not evident until 3 h and peaked at more than 10 h after hCG. Ovulation began between 8 and 10 h after hCG and 83% of animals had ovulated within 12 h.

Doses of 90 or 1800 µg indomethacin given together with hCG substantially inhibited ovulation and PG synthesis, but only the higher dose inhibited the hCG-induced elevation of progesterone and testosterone synthesis; hCG-induced oestradiol synthesis was not affected by either dose of indomethacin.

We conclude that the peak of PG synthesis after hCG treatment related closely to the timing of ovulation; the steroidogenic response to hCG was not blocked by doses of indomethacin sufficient to inhibit synthesis of PGE-2 and PGF-2α by more than 80%.

Introduction

Alterations in the ovarian follicle synthesis of cAMP, steroids, prostaglandins, ovarian weight and ovarian hyperaemia take place after gonadotrophin stimulation (Yang et al., 1973; LeMaire et al., 1975; Shaefer & Weidenfeld, 1975; Armstrong et al., 1976).

Each of these factors may play a role in processes culminating in ovulation, which is a highly synchronized event of many inter-dependent parts. Prostaglandins (PG) appear to have a mandatory role in rupture of follicles, acting directly or modulating the effects of other hormones (Armstrong & Grinwich, 1972; LeMaire et al., 1973, 1975).

Ovulation blocked by indomethacin can be restored by exogenous PGs (Tsafriri et al., 1973; Wallach et al., 1975). However, whether PGs are involved in steroidogenesis or if steroidogenesis is an obligatory step in the ovulation process are considered unlikely because indomethacin prevents ovulation without affecting luteinization of follicles and steroid production (Grinwich et al., 1972; O’Grady et al., 1972). These results were, however, questioned by Mori et al. (1980) who suggested that PGs mediate the actions of hCG on ovulation through two mechanisms which operate at different stages of the preovulatory process. They showed that, in rats treated with PMSG and hCG, the minimum effective dose of indomethacin (90 µg) which blocked ovulation when administered simultaneously with hCG also abolished the hCG-induced elevation of plasma progesterone and

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testosterone concentrations which peaked at about 3 h; the oestradiol surge was not affected. As the time after hCG elapsed, administration of 90 μg indomethacin was less effective at inhibiting ovulation, yet much higher doses of indomethacin would still block ovulation even when given 10–11 h after hCG. Mori et al. (1980) suggested that the initial steroidogenesis was a sensitive response to small increases in concentrations of PGs and was blocked by small doses of indomethacin, whereas closer to ovulation a direct effect of PGs on the follicle resulted from exposure to high concentrations and required large doses of indomethacin to block it. These conclusions were drawn from data in which PGs were not measured and ova were collected only at a single time (18 h) after hCG. To clarify the discrepancy between this and earlier studies we investigated the kinetic profiles of steroidogenesis, PG synthesis and ovulation with time after hCG, and the effect of low and high doses of indomethacin.

Materials and Methods

Animals

Wistar rats were bred in the Southampton University Medical School Animal House and used at 25–28 days of age and 45–60 g body weight. They were housed under constant conditions of lighting (lights on 06:30–20:30 h). Radiochemicals were obtained from Amersham, Bucks, U.K. Non-radioactive PGs (PGE-2 and PGF-2α) were gifts from Upjohn, (Kalamazoo, MI, U.S.A.). Non-radioactive steroids, indomethacin, PMSG (1000 i.u./mg protein) and hCG (2500 i.u./mg protein) were supplied by Sigma (St Louis, MO, U.S.A.).

Hormone and drug treatment. All animals in this study were injected subcutaneously with 5 i.u. PMSG in 0.2 ml saline (9 g NaCl/l). After 56 h each rat received intravenous saline or 10 i.u. hCG alone or simultaneously with indomethacin. Indomethacin was prepared according to Mori et al. (1980) and diluted with distilled water before use to give a concentration of 90 or 1800 μg in 0.2 ml at pH 7.4.

Induction of ovulation. Rats treated with PMSG and saline or hCG or hCG plus indomethacin were killed at 0, 3, 5, 6, 8, 10, 12 or 18 h after treatment. The oviducts and uteri were removed and the uterine contents were flushed into a Petri dish with ~1 ml saline and tubal and intrauterine ova were counted under a dissecting microscope.

Effect of indomethacin on hCG-induced PG and steroid production by whole follicles. Rats treated with PMSG were killed 0, 1, 3, 5, 8, 10, 18 or 24 h after injection of saline, hCG (10 i.u.) or hCG plus indomethacin (1800 μg/rat). The largest follicles were placed in 1 ml freshly prepared pre-oxygenated solution consisting of (g/litre): NaCl 5.54; KCl 0.35; MgSO4.7H2O 0.29; CaCl2 0.28; KH2PO4 0.16; NaHCO3 2.1; glucose 2.1, pH 7.4. The incubation technique used was as described by Marsh et al. (1974); follicles were preincubated for 30 min at 37°C in a shaking water bath and then transferred to 1 ml fresh Krebs' solution and the incubations were continued for 1 h at 37°C under the same conditions. At the end of incubation, the medium and tissue were frozen at −20°C until steroid and PG contents were measured.

In a second series of experiments, rats treated with PMSG were injected with hCG or hCG plus 90 μg indomethacin per rat. Steroid synthesis was assessed in follicles removed 3 h after treatment and PG synthesis in follicles removed at 8 h.

Radioimmunoassays

Prostaglandins. PGE-2 and PGF-2α were assayed largely as published by Hillier et al. (1985) and modified as follows.

The content of PGE-2 and PGF-2α in 0.1 and 0.2 ml of appropriately diluted Krebs' solution was measured by radioimmunoassay without extraction or purification. Previous tests showed that the antisera utilized to measure PGE-2 and PGF-2α exhibited low cross-reactivities to most other major PGs containing 2 double bonds and their metabolites.

Prior experiments had also confirmed (data not shown) that extraction of the Krebs' incubate with ether or by purifying on octadecasilyl silica columns according to the method of Powell (1980) gave estimates of PGE-2 and PGF-2α that were not significantly different from those in unextracted Krebs' solution. The intra-assay coefficient of variation (n = 6) was 7.9% (PGF-2α) and 10.2% (PGE-2) and the interassay coefficient of variation (n = 8) was 9.1% (PGF-2α) and 9.7% (PGE-2).

Protein was assayed in digested tissue by the modified method of Lowry et al. (1951).

Steroids. Steroid concentrations in duplicate ether extracts of 100 μl Krebs' incubate were determined using antisera raised in New Zealand White rabbits against progesterone-11-carboxymethylxime–BSA, testosterone-11-carboxymethylxime–BSA or oestradiol-6-carboxymethylxime–BSA (antigens were obtained from Steraloids Inc., Wilton, NH, U.S.A.). The antisera had titres of 1:8000, 1:8000 and 1:22 000, respectively.

The minimum sensitivity of the progesterone antiserum was 2 × 10^{-13} mol/tube; it had a cross-reactivity of 5% with 20α-dihydroprogesterone, 2% with pregnenolone and <1% with other steroids. The intra-assay coefficient of variation (CV) was 6.5% and the inter-assay CV was 12.2%.
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The minimum sensitivity of the testosterone antiserum was $2 \times 10^{-13}$ mol/tube; it had a cross-reactivity of 50% with dihydrotestosterone, and <1% with other steroids. The intra-assay CV was 6-9% and the interassay CV was 11%.

The minimum sensitivity of the oestradiol antiserum was $5 \times 10^{-14}$ mol/tube; it had a cross-reactivity of 35% with oestrone, 5% with oestradiol and <1% with other steroids. The intra-assay CV was 8-9% and the inter-assay CV was 14-9%.

For all the assays, the extracted steroid or standards was incubated for 3-4 h with the antiserum and tritiated label ([1,2,3,4-3H]progesterone, [3H]testosterone or [3H]oestradiol: Amersham, Bucks, U.K.) and free and bound steroid were separated using dextran-coated charcoal. The bound fraction was counted. Data were analysed using a logit fit of the standard curve; values which fell beyond the 10% limits on the line were considered to be undetectable.

Statistical tests

The results, expressed as a mean ± s.e.m., were analysed by Wilcoxon’s rank sum test.

Results

Time of ovulation after hCG

Table 1 shows that at 8 h after hCG no animals had ovulated but at 10 h 50% of the animals had ovulated with 3-6 ± 0-1 ova shed per ovulating rat. By 12 h 83% had ovulated with 10-8 ± 0-9 ova released per ovulating rat.

<table>
<thead>
<tr>
<th>Time after hCG injection (h)</th>
<th>No. of rats</th>
<th>No. of rats ovulating</th>
<th>No. of ova released per animal ovulating</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-7</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>6</td>
<td>3-6 ± 0-1</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>10</td>
<td>10-8 ± 0-9</td>
</tr>
<tr>
<td>18</td>
<td>18</td>
<td>18</td>
<td>14-8 ± 1-0</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.

Effect of 90 or 1800 µg indomethacin on hCG-induced ovulation

In other series of experiments hCG caused all animals to ovulate (13/13) with 13-3 ± 1-8 ova shed per animal. Indomethacin (90 µg) reduced this to 4/15 animals ovulating with 3-5 ± 1-1 ova shed per ovulating animal, and with 1800 µg indomethacin 0/15 animals ovulated. Ovulation was assessed 18 h after hCG.

Effect of hCG and hCG plus 1800 µg indomethacin on synthesis of PGs and steroids by ovarian follicles

Table 2 shows that hCG-injection resulted in a significant increase in the follicular synthesis of PGE-2 and PGF-2α. There was a time lag of 1-3 h after hCG before PG synthesis increased. The greatest increment in PG synthesis occurred between 5 and 8 h after hCG and peak synthesis occurred around 10 h, when the first ova were shed. Between 5 and 8 h PGF-2α accumulation increased about 2-5-fold while that of PGE-2 increased about 5-fold. PGF-2α synthesis exceeded PGE-2 synthesis up to 5 h after hCG but at 8 and 10 h PGE-2 synthesis exceeded that of
Table 2. Accumulation of PGE-2 and PGF-2α (pmol/mg protein) by incubated follicles from PMSG-primed rats at different times after intravenous administration of saline, hCG or hCG plus indomethacin (I, 1800 µg)

<table>
<thead>
<tr>
<th>Hours</th>
<th>No. of rats</th>
<th>PGE-2</th>
<th>hCG</th>
<th>hCG + I</th>
<th>PGF-2α</th>
<th>hCG</th>
<th>hCG + I</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td>304 ± 45 am</td>
<td>234 ± 22 am</td>
<td>269 ± 27 am</td>
<td>317 ± 17 am</td>
<td>484 ± 19 am</td>
<td>444 ± 21 am</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>318 ± 22 am</td>
<td>304 ± 19 am</td>
<td>220 ± 17 am</td>
<td>383 ± 19 am</td>
<td>684 ± 102 am</td>
<td>395 ± 14 am</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>346 ± 15 am</td>
<td>304 ± 26 am</td>
<td>220 ± 17 am</td>
<td>374 ± 29 am</td>
<td>4506 ± 473 am</td>
<td>689 ± 54 am</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>299 ± 31 am</td>
<td>1493 ± 301 cn</td>
<td>493 ± 78 am</td>
<td>316 ± 35 am</td>
<td>1817 ± 234 am</td>
<td>512 ± 41 am</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>268 ± 21 am</td>
<td>7598 ± 583 dn</td>
<td>669 ± 102 am</td>
<td>398 ± 25 am</td>
<td>6934 ± 483 am</td>
<td>823 ± 87 am</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>369 ± 34 am</td>
<td>10948 ± 1238 am</td>
<td>1234 ± 384 do</td>
<td>1175 ± 123 am</td>
<td>1902 ± 156 am</td>
<td>2704 ± 237 am</td>
</tr>
<tr>
<td>18</td>
<td>12</td>
<td>273 ± 29 am</td>
<td>534 ± 117 bm</td>
<td>613 ± 94 am</td>
<td>424 ± 18 am</td>
<td>778 ± 92 bm</td>
<td>689 ± 67 am</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td>342 ± 14 am</td>
<td>399 ± 22 am</td>
<td>401 ± 24 am</td>
<td>458 ± 32 am</td>
<td>462 ± 45 am</td>
<td>34 am</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
Values within a vertical column (a–e) or horizontal row (m–o) without a common superscript letter are significantly different (P < 0.01, Wilcoxon’s rank sum test).

Table 3. Accumulation of progesterone, testosterone and oestradiol (pmol/mg protein) by incubated follicles from PMSG-primed rats at different times after saline, hCG or hCG plus indomethacin (I, 1800 µg)

<table>
<thead>
<tr>
<th>Hours</th>
<th>No. of rats</th>
<th>Progesterone</th>
<th>hCG</th>
<th>hCG + I</th>
<th>Testosterone</th>
<th>hCG</th>
<th>hCG + I</th>
<th>Oestradiol</th>
<th>hCG</th>
<th>hCG + I</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td>132 ± 6 am</td>
<td>135 ± 3 am</td>
<td>141 ± 3 am</td>
<td>138 ± 13 am</td>
<td>142 ± 14 am</td>
<td>151 ± 18 am</td>
<td>168 ± 11 am</td>
<td>158 ± 19 am</td>
<td>162 ± 14 am</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>153 ± 10 am</td>
<td>633 ± 11 am</td>
<td>509 ± 11 am</td>
<td>139 ± 23 am</td>
<td>497 ± 29 am</td>
<td>373 ± 14 am</td>
<td>175 ± 14 am</td>
<td>482 ± 33 am</td>
<td>492 ± 29 am</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>178 ± 14 am</td>
<td>1561 ± 1237 am</td>
<td>1328 ± 1232 am</td>
<td>192 ± 18 am</td>
<td>857 ± 165 am</td>
<td>542 ± 42 am</td>
<td>182 ± 16 am</td>
<td>586 ± 47 am</td>
<td>671 ± 51 am</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>120 ± 13 am</td>
<td>1132 ± 152 am</td>
<td>943 ± 86 am</td>
<td>123 ± 15 am</td>
<td>683 ± 63 am</td>
<td>488 ± 74 am</td>
<td>179 ± 17 am</td>
<td>301 ± 20 am</td>
<td>313 ± 29 am</td>
</tr>
<tr>
<td>10</td>
<td>15</td>
<td>115 ± 23 am</td>
<td>293 ± 16 am</td>
<td>262 ± 18 am</td>
<td>138 ± 18 am</td>
<td>223 ± 15 am</td>
<td>284 ± 17 am</td>
<td>169 ± 10 am</td>
<td>142 ± 8 am</td>
<td>136 ± 8 am</td>
</tr>
<tr>
<td>18</td>
<td>12</td>
<td>143 ± 21 am</td>
<td>218 ± 18 am</td>
<td>242 ± 11 am</td>
<td>196 ± 11 am</td>
<td>121 ± 10 am</td>
<td>117 ± 12 am</td>
<td>172 ± 8 am</td>
<td>153 ± 13 am</td>
<td>179 ± 7 am</td>
</tr>
<tr>
<td>24</td>
<td>6</td>
<td>154 ± 19 am</td>
<td>183 ± 11 am</td>
<td>187 ± 10 am</td>
<td>199 ± 14 am</td>
<td>137 ± 18 am</td>
<td>129 ± 12 am</td>
<td>155 ± 8 am</td>
<td>156 ± 9 am</td>
<td>161 ± 6 am</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m.
Values within a vertical column (a–e) or horizontal row (m–o) without a common superscript letter are significantly different (P < 0.01, except at * when P < 0.05, Wilcoxon’s rank sum test).

PGF-2α accumulation (P < 0.01). By 24 h both PGs had returned to basal levels of synthesis. Indomethacin (1800 µg) decreased PG synthesis markedly although suppression was not complete, particularly from 5 to 10 h after hCG. At 18 and 24 h after indomethacin plus hCG, PG synthesis was not different from that after hCG treatment alone.

Table 3 shows the synthesis of progesterone, testosterone and oestradiol in incubates of follicles after hCG or hCG plus indomethacin: hCG stimulated the synthesis of all the steroids measured and the peak of stimulation was at about 3 h. The relative degree of stimulation was progesterone > testosterone > oestradiol. The stimulation of synthesis had substantially declined by 10 h after
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treatment. Indomethacin (1800 µg) inhibited the hCG-induced increases in progesterone and testosterone at 3 and 5 h ($P < 0.01$). The percentage decrease at 3 h was 15% for progesterone and 37% for testosterone, and at 5 h was 17% for progesterone and 29% for testosterone. The oestriadiol synthesis induced by hCG was not influenced by indomethacin.

In the second series of experiments ($n = 6$) 90 µg indomethacin did not alter hCG-induced steroid synthesis by follicles harvested after treatment (data not shown). PGE-2 and PGF-2α synthesis under the influence of 90 µg indomethacin fell from 8292 ± 817 and 5079 ± 390 to 938 ± 248 and 898 ± 183 pmol/mg protein respectively.

Discussion

These data confirm the widely reported facts that hCG administration to PMSG-primed immature rats sets in motion a chain of events within the follicle that lead to ovulation. However, it is less certain whether the attendant changes in steroidogenesis and prostaglandin elevation and follicular rupture are in any way inter-dependent with one event obligatorily determining another.

We could not support the claim of Mori et al. (1980) that the early hCG-induced steroidogenic response was a sensitive PG-dependent process. The high dose of indomethacin inhibited PG synthesis substantially at all times (Table 2) and, although significant, inhibited progesterone synthesis by <20% and testosterone synthesis by <40%. Moreover, the low dose of indomethacin (90 µg) also resulted in a substantial inhibition of PG synthesis (89% for PGE-2 and 83% for PGF-2α) without significantly affecting steroid synthesis. The inhibitory effect of 1800 µg indomethacin on progesterone and testosterone synthesis was also considerably less in this study than in that of Mori et al. (1980).

A comparison of the time profile of response of PG and steroids to hCG indicates marked differences. At 1 h after hCG all steroids in the incubates were significantly increased but raised prostaglandin concentrations were not significant until 3 h after hCG. The inhibition of steroidogenesis by high doses of indomethacin is therefore probably due to factors other than inhibition of synthesis of PGE-2 and PGF-2α synthesis. Although in this work we have made no attempt to show that the hCG-induced increase in PG synthesis is independent of the elevation in steroid synthesis, others have shown this to be so (Armstrong et al., 1976).

Assessment of the number of rats ovulating 18 h after hCG (10 i.u.) and the number of ova shed per rat (14-8) in this work (Table 1) compares very closely with the data of Mori et al. (1980). Our results also show that release of ova began abruptly between 8 and 10 h after hCG (no ova were present at the 8-h time point), and by 10 h 50% of animals had begun to ovulate. Between 10 and 12 h almost all had started to ovulate and the majority of ova were shed at this time.

The incontrovertable effect of indomethacin on ovulation blockade clearly suggests that PGs are implicated in the process. Of the many PGs or lipoygenase-dependent products of arachidonate metabolism that may be involved in ovulation we have measured only two, PGE-2 and PGF-2α. We have shown in sequential studies (Table 2) that follicular synthesis of PGE-2 and PGF-2α peaks about 10 h after hCG, showing a very long time course of response, especially when it is known that PGs are not stored in tissue but are rapidly synthesized on demand. The onset of ovulation relates closely to the peak levels of PG synthesis.

Mori et al. (1980) showed that 1800 µg indomethacin will actively inhibit ovulation even when given 12–13 h after hCG. By this time the majority of rats in our study had ovulated and the peak of PGE-2 and PGF-2α synthesis had also occurred. Because Mori et al. (1980) did not measure PG synthesis at different times after hCG and did not assess the precise timing of ovulation, it is not possible to conclude whether the effects they observed were due to PG inhibition or perhaps to an effect unrelated to PGs.

Espey & Coons (1976) have shown that indomethacin inhibits collagenolytic activity in rabbit follicles. They were able to discount an effect of PGE-2 on collagenolytic activity, but it may be that
other prostanoids are involved in this aspect of follicular rupture rather than the mechanism of action of indomethacin not involving inhibition of prostaglandin synthesis. There is also some evidence that indomethacin will inhibit protein synthesis (Reeds & Palmer, 1983) and theoretically high doses may therefore interfere with ovulation at a late stage in the ovulatory process by inhibiting protein-synthesis dependent lytic processes in the follicle wall. Specific studies on follicle proteinases are clearly required to clarify these points.

The failure of indomethacin (1800 µg) to inhibit oestradiol synthesis, while reducing progesterone and testosterone accumulation, could suggest that the residual levels of testosterone present were perhaps high enough to maintain oestrogen synthesis.

While the present study suggests that the inhibition of ovarian PG synthesis may be one component of the action of indomethacin, it may have many actions which are unrelated to inhibition of PG synthesis and caution is therefore required in its use for dissecting out purely PG-related processes. The use of indomethacin without measuring PG synthesis levels is probably of limited value.

We thank the World Health Organisation for financial support.

References


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