EFFECT OF AMINOGLUTETHIMIDE PHOSPHATE ON IN VITRO OESTRADIOL STIMULATED PROGESTERONE SYNTHESIS BY RABBIT CORPORA LUTEA

By
Gene B. Fuller, Barry M. Markaverich1) and William C. Hobson

ABSTRACT
Slices of rabbit corpora lutea were incubated for 3 and 8 h with oestradiol-17β in the presence or absence of aminoglutethimide phosphate (AGP)2) in order to identify a possible site(s) of action of oestrogen on progesterone synthesis. Oestradiol was unable to increase progesterone biosynthesis above controls in luteal tissue exposed to AGP during either incubation period. AGP alone significantly reduced progesterone concentrations and synthesis from [14C]acetate at both time periods while oestradiol alone increased progesterone mass at 8 h. Changes in 20α-OH progesterone typically paralleled those of progesterone at 3 h but no effect of the inhibitor was seen at 8 h. Except for an increase in 14C-incorporation into cholesterol esters in the AGP-treated groups at 8 h the concentration or synthesis of sterols from labelled acetate did not respond to treatment.

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2) Aminoglutethimide phosphate kindly donated by Dr. J. J. Chart, CIBA Pharmaceutical Co., Summit, N. J.

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A preliminary report of some of this work was presented at the Ninth Annual Meeting of the Society for the Study of Reproduction, 1976.
These results suggested that cholesterol side chain cleavage and cholesteryl synthesis are not major in vitro sites of action of oestradiol in the rabbit corpus luteum.

Oestrogen is considered to be the major luteotropic factor in the rabbit. Nearly four decades ago Robson (1939) reported that oestrogen was capable of maintaining the normal morphology of corpora lutea in hypophysectomized rabbits. More recently, the trophic effect of oestradiol was confirmed in hypophysectomized rabbits (Spies & Quadri 1967; Spies et al. 1968), in rabbits with x-irradiated ovaries (Keyes & Nalbandov 1967) and in rabbits treated with anti-gonadotrophic sera (Spies & Quadri 1967) or with an anti-oestrogen (Labhsetwar 1971). Oestradiol has also been found to stimulate progesterone synthesis in slices of rabbit luteal tissue in vitro (Fuller & Hansel 1971). Furthermore, the elucidation of a cytoplasmic oestrogen receptor in the rabbit corpus luteum (Lee et al. 1972), provided substantive evidence for this gland as an oestrogen target tissue. Nevertheless, the mechanism(s) by which oestradiol induces progesterone synthesis is not known.

We have investigated the site of oestradiol action in rabbit corpora lutea through a series of 3 and 8 h in vitro experiments utilizing various metabolic inhibitors. These two incubation intervals were based on results of a time study in our laboratory examining incubation periods from 1 to 16 h which revealed that the rate of oestradiol-induced progesterone synthesis increased slowly through 3 h then increased sharply to a maximum at 8 h (unpublished observations).

Relatively specific inhibitors such as aminogluthethimide, AY-9944, cyanoacetone and oxymetholone have been used with moderate success to study the regulation of steroidogenesis in gonadal and adrenal tissue in vitro (Kowal 1969; Armstrong et al. 1970; Depp et al. 1973). This report describes the results of studies with aminogluthethimide phosphate (AGP), a competitive inhibitor of the conversion of cholesterol to pregnenolone. AGP reportedly blocks the initial 20α-hydroxylation of cholesterol (Kahnt & Neher 1966; Cohen 1968) which has been suggested to be a rate-limiting step in steroidogenesis (Stone & Hechter 1954; Hall & Koritz 1965).

**MATERIALS AND METHODS**

**Chemicals**

All solvents were of reagent grade and were distilled prior to use. Aminogluthethimide phosphate (Elipten) was obtained from CIBA Pharmaceutical Co., Summit, N. J. [14C]Sodium acetate (59 mCi/mM), [3H]progesterone (16 Ci/mM), [3H]20α-hydroxyprogesterone (21 Ci/mM) and [3H]cholesterol (17 Ci/mM) were obtained from New England Nuclear, Boston, Mass., and re-chromatographed prior to use. Oestradiol-17β and 2,7-dichlorofluorescein were obtained from Sigma, Saint Louis, Mo.
**Animals and treatment**

Sexually mature Dutch-Belted X New Zealand female rabbits were utilized in all studies. Does mated to fertile bucks were sacrificed by cervical dislocation on day 11 of pregnancy. Corpora lutea (6–9 per rabbit) were dissected from extraneous ovarian tissues rinsed in chilled saline (0.9%), sliced and 30–40 mg of tissue randomly distributed to incubation vials. Each vial contained 3 ml of Krebs-Ringer bicarbonate buffer (gassed to pH 7.4 with 95% O₂:5% CO₂), 2 mg glucose/ml, 30 mmoles of nicotinamide and 10 μCi [¹⁴C]acetate. Incubations were carried out in a Dubnoff metabolic shaker at 37°C for 3 or 8 h. To solubilize oestradiol-17β in the aqueous incubation medium, the steroid was first transferred to the protein fractions of hypophysectomized-ovariectomized rabbit during a 3-h pre-incubation period as previously described by Fuller & Hansel (1971). In all incubations 0.1 ml of serum or serum-oestradiol was added to the buffered medium. During subsequent *in vitro* studies oestradiol has been added to vials in ethanol and the ethanol evaporated prior to addition of the medium. No differences in the response to oestrogen have been noted (unpublished observations). Incubations were terminated by freezing on dry ice and vials were stored at −20°C for subsequent analysis.

Two 3-h and two 8-h incubations were conducted. Each incubation utilized luteal tissue from 6–8 rabbits and contained the following treatment groups: incubated controls, oestradiol-17β, AGP and oestradiol-17β + AGP. Aminoglutethimide phosphate (10⁻³ M) was dissolved in 0.9% saline and added to the incubation medium. This level of the inhibitor has been shown to reversibly reduce *in vitro* steroid output 85–95% by adrenal and ovarian cell cultures (Kowal 1969; Solomon & Sherman 1976) or by tissue slices of adrenals and ovaries (Dexter et al. 1967; Wilks et al. 1970). Aminoglutethimide concentrations of 10⁻³ M or greater have been shown to have no adverse effect on cell growth, RNA and protein synthesis, enzyme activity or cholesterol synthesis (Kowal 1969; Solomon & Sherman 1976). Oestradiol was added to the medium as described above at a dose level of 5 μg/vial. This amount was previously found to give optimum stimulation of progesterone synthesis under our *in vitro* conditions. Synthesis of steroids and sterols was expressed as μg/g of luteal tissue (wet weight), respectively, while [¹⁴C]acetate-incorporation was expressed as DPM/mg tissue (wet weight). Data were analyzed statistically utilizing analysis of variance and Duncan’s New Multiple Range Test (Steel & Torrie 1960). The comparisons tested were as follows: incubated control vs. oestradiol; incubated control vs. AGP; incubated control vs. AGP + oestradiol; AGP vs. oestradiol + AGP.

**Extraction and separation of steroids**

Following the addition of 10 000 cpm of [³H]progesterone, [³H]20α-hydroxyprogesterone and [³H]cholesterol, tissues were ground in the incubation medium and the homogenates extracted in 4 × 15 ml volumes of diethyl ether. Ether extracts were dried down under vacuum and submitted to two-dimensional thin-layer chromatography as previously described by Armstrong et al. (1964). The steroids and sterols (sprayed with 2,7-dichlorofluorescein) were viewed under UV light, eluted from the silica gel, and aliquoted for radioactivity determinations. Progesterone and 20α-hydroxyprogesterone were measured by gas-liquid chromatography as previously described (Fuller & Hansel 1970). Sterols were quantitated spectrophotometrically using a modified Lieberman-Buchard reaction. The recovery of the tritiated progesterone, 20α-hydroxyprogesterone and cholesterol allowed for correction, due to procedural losses, of both mass and ¹⁴C-radioactivity. Concentration and ¹⁴C-incorporation into steroid esters were not corrected for procedural losses.
Table 1.
Effect of AGP on progestin and sterol biosynthesis in rabbit corpora lutea during a 3-h incubation period.

<table>
<thead>
<tr>
<th></th>
<th>Progesterone</th>
<th>20α-ol</th>
<th>Cholesterol</th>
<th>Cholesterol ester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/g***</td>
<td>DPM-[¹⁴C]/mg</td>
<td>µg/g</td>
<td>DPM-[¹⁴C]/mg (x 10⁻²)</td>
</tr>
<tr>
<td>Incub. cont. (7)**</td>
<td>44.8 ± 2.2</td>
<td>598 ± 32</td>
<td>5.8 ± 1.3</td>
<td>84 ± 14</td>
</tr>
<tr>
<td>Oe₂ (6)</td>
<td>49.5 ± 2.6</td>
<td>542 ± 60</td>
<td>1.3 ± 0.3*</td>
<td>49 ± 20*</td>
</tr>
<tr>
<td>AGP (8)</td>
<td>17.0 ± 1.8</td>
<td>37 ± 10*</td>
<td>2.6 ± 0.7</td>
<td>55 ± 22*</td>
</tr>
<tr>
<td>Oe₂ + AGP (6)</td>
<td>17.6 ± 1.4*</td>
<td>38 ± 8*</td>
<td>3.9 ± 0.6</td>
<td>127 ± 25</td>
</tr>
</tbody>
</table>

* Different from incubated control (P < 0.05).
** Value in brackets is the number of incubation vials.
*** µg/g, DPM-[¹⁴C]/mg or mg/g are expressed as “units” per mg or g of luteal tissue (wet weight), values represent mean ± SEM.
Identification of products

The authenticity of radioactive steroids synthesized from labelled acetate during incubations was determined by comparison of thin-layer chromatographic mobilities to authentic progesterone, 20α-hydroxyprogesterone (20α-ol), cholesterol and cholesterol laurate standards. The radiochemical purity of progesterone and 20α-hydroxyprogesterone isolated by these procedures was verified to be 98–100% by radiochromatogram scanning.

RESULTS

3-h incubation

Addition of aminoglutethimide phosphate (AGP) to the incubation medium resulted in 60% reduction (P < 0.05) in progesterone concentration (μg/g) and decreased (P < 0.05) the incorporation of [14C]acetate into this steroid to 7% of incubated control values (Table 1). Although oestradiol alone maintained 14C-incorporation into progesterone, and slightly increased concentration (0.1 > P > 0.05) it could not overcome the AGP block of progesterone synthesis (P < 0.05). Similar, AGP treatment reduced both the concentrations of 20α-hydroxyprogesterone and its synthesis from 14C-labelled precursors (P < 0.05) when compared with incubated controls. Although 20α-ol concentration in the oestradiol + AGP treatment groups was not different from incubated control values, 14C-incorporation into this metabolite was greatly reduced (P < 0.05). Except for an increase (P < 0.05) in 14C-incorporation into cholesterol esters in AGP treated groups, the concentration or synthesis of esterified sterols from [14C]acetate did not respond to treatment.

8-h incubation

The inhibitory effects of AGP on progesterone synthesis were more pronounced following the 8-h incubation period as mass and 14C-incorporation were reduced by 81 and 96% of the incubated control values, respectively (Table 2). This inhibition of progesterone synthesis by AGP was observed both in the presence and absence of oestradiol. Oestradiol alone, increased (P < 0.05) progesterone synthesis approximately 14 μg/g above incubated controls, but this was not reflected by [14C]acetate incorporation into progesterone. Surprisingly, no significant effect of AGP on the 20α-hydroxylated metabolite was found. When incorporation of 14C into 20α-ol was compared with incorporation into progesterone it was obvious that a much larger portion of the 20α-ol pool came from labelled precursors. The failure of oestradiol to overcome the AGP inhibition and stimulate progesterone synthesis was consistent with the results of the 3-h incubations. With the exception of a peculiar oestrogen-induced increase (P < 0.05) in 14C-labelled cholesterol esters in the oestradiol + AGP treated vials no treatment effects were observed in the free or esterified fractions at 8 h.
Effect of AGP on progestin and sterol biosynthesis in rabbit corpora lutea during a 8-h incubation period.

<table>
<thead>
<tr>
<th></th>
<th>Progesterone</th>
<th>20α-ol</th>
<th>Cholesterol</th>
<th>Cholesterol ester</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/g**</td>
<td>DPM-[14C]/mg</td>
<td>µg/g</td>
<td>DPM-[14C]/mg</td>
</tr>
<tr>
<td>Incub. cont. (8)**</td>
<td>31.9 ± 3.9</td>
<td>739 ± 75</td>
<td>4.7 ± 1.1</td>
<td>195 ± 30</td>
</tr>
<tr>
<td>Oe₂ (6)</td>
<td>45.8 ± 2.2*</td>
<td>778 ± 74</td>
<td>5.5 ± 1.1</td>
<td>285 ± 50</td>
</tr>
<tr>
<td>AGP (8)</td>
<td>6.1 ± 0.6*</td>
<td>32 ± 10*</td>
<td>4.5 ± 1.9</td>
<td>132 ± 58</td>
</tr>
<tr>
<td>Oe₂ + AGP (6)</td>
<td>6.6 ± 0.8*</td>
<td>31 ± 10*</td>
<td>1.4 ± 0.3</td>
<td>153 ± 33</td>
</tr>
</tbody>
</table>

* Different from incubated control (P < 0.05).
** Value in brackets is the number of incubation vials.
*** µg/g, DPM-[14C]/mg or mg/g are expressed as “units” per mg or g of luteal tissue (wet weight), values represent mean ± SEM.
DISCUSSION

This study was based on the hypothesis that oestradiol would stimulate progesterone synthesis in the presence of AGP if the conversion of cholesterol to pregnenolone was a primary site of oestrogen action in the rabbit corpus luteum. Conversely a failure to overcome the block would tentatively eliminate oestrogen induction of the 20α-hydroxycholesterol enzymes as a site of action.

The results demonstrate that the addition of oestradiol to the medium failed to stimulate progesterone synthesis by rabbit luteal tissue slices exposed to AGP during either a 3 or 8 h incubation period (Tables 1 and 2). Similar findings were reported by Fuller & Hansel (1971) following in vivo pre-treatment of rabbits with the inhibitor. Failure of oestrogen to overcome the AGP inhibition suggests that oestradiol does not regulate the 20α-hydroxycholesterol dehydrogenase enzyme or subsequent enzymes involved in the utilization of cholesterol for pregnenolone synthesis. The data further indicate that oestradiol does not increase the concentration of enzyme substrates. However, it is possible that if under our in vitro conditions the amount of competitive inhibitor was excessive it would be difficult to bypass the block in spite of increased enzyme activity. Since the concentration of AGP used in this study was in the low part of the range found to give adequate inhibition of in vitro steroidogenesis (Kowal 1969) it seems unlikely that an oestrogen-induced increase of enzyme activity or concentration would have failed to be reflected by a change in the production of progesterone. Interestingly, this site of action has been proposed for LH in ovarian tissues (Behrman & Armstrong 1969; Behrman et al. 1972).

As antipated, the addition of AGP to the medium reduced both progesterone concentration and progesterone synthesis from radioactive acetate. These findings indicated that AGP reduced the conversion of pre-existing precursor into progesterone and blocked the incorporation of de novo synthesized precursors into the steroid. These results are in agreement with previous studies in rat ovarian tissue and rabbit and bovine corpora lutea (Wilks et al. 1970; Fuller & Hansel 1971). There were no significant changes in 20α-OH-progesterone except for a decrease in mass and incorporation in the AGP group at 3 h. Since 20α-ol is thought to be solely derived from progesterone the greater incorporation of 14C into 20α-OH, relative to progesterone, in the presence of AGP presents an enigma. This finding indicates that the pools of 20α-ol and progesterone do not equilibrate and further suggests that there was some early conversion of labelled acetate into progesterone before the effect of the inhibitor became complete. Another possible explanation would be an alternate pathway by which cholesterol and progesterone are bypassed in the biosynthesis of 20α-ol. In vivo metabolism studies in the pregnant mare (Bhavnani & Short 1973) and in vitro studies with rat adrenals (Lommer et al.
indicate that cholesterol may not be an obligatory intermediate for steroid biosynthesis.

One possible site of action of oestradiol which may be eliminated by this study was on the synthesis of cholesterol. If the site of action of oestradiol was on cholesterol synthesis an increase in this sterol would be expected in the AGP and oestradiol treatment groups. A combination of the AGP blocked conversion of cholesterol to pregnenolone plus oestradiol stimulated cholesterol synthesis would greatly increase the sterol pool. This did not occur at either incubation period. Subsequent studies in our laboratory with AY-9944, an inhibitor of cholesterol synthesis, have tentatively confirmed that oestrogen does not appreciably stimulate the biosynthesis of cholesterol and that oestradiol-induced steroidogenesis is not directly dependent on *de novo* sterol biosynthesis (unpublished observation).

Little can be inferred from these data concerning active sites subsequent to cholesterol side chain cleavage. If oestradiol was acting a sites after cholesterol hydroxylation one might have anticipated the hormone would overcome the inhibitor, resulting in increased progesterone synthesis. However, as precursors subsequent to cholesterol in the pathway are highly dependent on the 20α-hydroxylation reaction, biosynthesis from cholesterol would be effectively blocked by AGP.

In conclusion, this study substantiated that AGP is a potent inhibitor of progesterone synthesis in rabbit corpora lutea in *vitro* during 3 and 8 h incubation periods. This inhibition cannot be overcome by the addition of oestradiol to the incubation medium. Although these results alone cannot establish the site(s) of action of oestradiol in the biosynthetic pathway to progesterone, they do appear to eliminate cholesterol synthesis, and cholesterol side-chain cleavage as major in *vitro* sites of action of oestradiol in the rabbit corpus luteum.

REFERENCES


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