Effect of LH factors regulating ovarian cholesterol metabolism and progesterone synthesis in PMSG-primed immature rats

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Summary. Administration of an ovulatory dose of LH (10 µg, i.v.) to PMSG (4 i.u., s.c.)-primed immature rats increased ovarian pregnenolone levels 5-fold and ovarian progesterone levels 40-fold within 6 h and the levels, although fluctuating, remained elevated for 72 h. Serum progesterone levels mimicked those of the luteal phase in the normal cycle. The sustained increase in steroidogenesis was accompanied by a decrease in both basal and cAMP-stimulatable ovarian cholesterol ester hydrolase activity and a net increase in ovarian cholesterol ester content. Ovarian free cholesterol levels were essentially unchanged during the 72 h study. LH does not, therefore, chronically stimulate steroidogenesis by providing additional substrate for the steroidogenic enzymes through activating cholesterol ester hydrolase to bring about hydrolysis of cholesterol esters. Moreover, ovarian cholesterol esters are unlikely to be the primary source of cholesterol utilized to support luteal steroidogenesis. Studies with isolated mitochondria suggested that the mechanisms by which LH stimulated steroidogenesis were by (a) stimulating mitochondrial pregnenolone production probably by facilitating the intramitochondrial movement of cholesterol to the site of side-chain cleavage, and (b) promoting the metabolism of pregnenolone to progesterone.

Introduction

While the stimulation of ovarian progesterone production by luteinizing hormone (LH) is understood to be mediated through adenosine-3′,5′-monophosphate (cAMP) (Marsh, 1976; Sala, Dufau & Catt, 1979), the mechanism by which cAMP actually brings about and maintains increased progesterone production is poorly understood. Cholesterol is the obligatory precursor of progesterone and changes in either its cellular concentration or metabolism might, therefore, be expected to play a central role in regulating progesterone biosynthesis. The greatest increase in ovarian progesterone production occurs at the time of ovulation when the preovulatory LH surge initiates progesterone production by the corpus luteum. The purpose of this study was to attempt to correlate this chronic increase in ovarian progesterone production, induced by LH, with changes in ovarian cholesterol content and the biochemical conditions which might be expected to regulate the metabolism of cholesterol to progesterone.

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Materials and Methods

Animals

Immature Wistar strain female rats, 21–23 days old and weighing 30–45 g, were obtained from the Small Animal Breeding Station (University of Edinburgh). The animals were housed under constant temperature and lighting conditions and were allowed pelleted food and water ad libitum. At 23–25 days of age, follicular development was stimulated with a subcutaneous (s.c.) injection of 4 i.u. PMSG (Gestyl: Organon Laboratories Ltd, Crown House, Morden, Surrey, U.K.) in 0.2 ml saline (9 g NaCl/l). Follicular development initiated in this way mimics that of the mature animal in that, after an ovulatory stimulus, the induced ovulation closely resembles a spontaneous ovulation in terms of preovulatory serum oestradiol-17β and progesterone concentrations, number of follicles ovulated (5–7 per ovary) and post-ovulatory serum progesterone concentrations (Guillet & Rennels, 1964; Goff & Henderson, 1979). Ovulation was induced 2 days after PMSG priming by an intravenous (i.v.) injection through a tail vein of 10 µg LH (NIH-LH-S18) in 0.2 ml saline. Animals were killed by decapitation at selected times after LH administration. Trunk blood was collected and stored overnight at 4°C and the serum was collected and stored at −20°C until assayed for progesterone. Ovaries were excised, trimmed free of fat, weighed and homogenized individually in 3 ml chloroform/methanol (2:1 v/v) for sterol and steroid extraction, or pooled and homogenized at 4°C in 10 ml 0.25 M-sucrose (pH 7.0) for subsequent preparation of mitochondria or the 105 000 g supematant used in the assay of cholesterol ester hydrolase.

Preparation of mitochondria and mitochondrial incubations

Ovarian homogenates in 0.25 M-sucrose were centrifuged for 10 min at 650 g to sediment nuclei and cell debris. The supernatant was centrifuged at 8500 g for 15 min to sediment the mitochondrial fraction which was washed in 0.25 M-sucrose and centrifuged again. The final pellet was taken up in chloroform/methanol (2:1 v/v) for cholesterol and steroid extraction or in 10 mM-potassium phosphate buffer (pH 7.4) containing 5 mM-MgCl2 and 100 mM-sucrose for measurement of cholesterol side-chain cleavage activity and cytochrome P-450 content. Mitochondrial protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Mitochondrial cholesterol side-chain cleavage activity was determined at 37°C in 1 ml potassium phosphate buffer which also contained 0.2 mM-NADP+. The reaction was initiated with 10 mM-DL-isocitrate after the mitochondria had been preincubated at 37°C for 15 min with 100 µM-cholesterol or 100 µM-24-hydroxycholesterol added in 10 µl ethanol. Control incubations received 10 µl ethanol only. Aliquots (0.2 ml) were pipetted into 2 ml chloroform/methanol (2:1 v/v) and vortexed briefly to stop the reaction at 2, 5, 10 and 20 min after the addition of isocitrate. Final extraction of steroids into the chloroform/methanol was achieved by vortexing the samples for 90 sec. The aqueous layer was removed and discarded, and the organic phase was stored at −20°C until assayed for pregnenolone and progesterone. The efficiency of extraction was monitored by the addition of tritiated steroid and was >95% for both pregnenolone and progesterone.

Mitochondrial cytochrome P-450 content was determined spectrophotometrically as described by Omura & Sato (1964) by recording the difference spectrum between reduced cytochrome P-450 and its carbon monoxide complex. The concentration of cytochrome P-450 was calculated by using an extinction coefficient of 91 mM⁻¹ cm⁻¹ for the absorbance change at 450–490 nm. The reducing agent was sodium dithionite.

Cholesterol, cholesterol ester and cholesterol ester hydrolase determinations

Trace amounts of [14C]cholesterol and [14C]cholesteryl oleate were added to aliquots of the ovarian and mitochondrial chloroform/methanol extracts. Cholesterol and cholesterol ester were
separated by thin-layer chromatography on Silica Gel H-plates developed in a solvent system consisting of petroleum ether:diethyl ether:glacial acetic acid (75:24:1, by vol.). Cholesterol and cholesterol ester were located by using a Panax thin-layer radioactive scanner and were eluted from the silica gel with diethyl ether:acetone (1:1 v/v). The eluates were dried under N₂ and the residues were taken up in ethanol.

Cholesterol was measured by the fluorescence technique described by Gamble, Vaughan, Kruth & Avigan (1978). In this method cholesterol is specifically oxidized with the enzyme cholesterol oxidase (B. D. H. Chemicals Ltd, Poole, Dorset, U.K.) and the H₂O₂ generated further reacted with p-hydroxyphenylacetic acid in a reaction catalysed by horseradish peroxidase (Sigma Chemical Co., London, U.K.) to yield a stable fluorescent product. This technique is sensitive to as little as 100 ng cholesterol. Cholesterol ester was measured by liberating cholesterol during saponification in 10% potassium hydroxide in ethanol at 60°C and assaying the cholesterol by the method above. Recovery of cholesterol and cholesterol ester was >90% as monitored by added [¹⁴C]sterols.

Cholesterol ester hydrolase activity in the 105 000 gₛ supernatant of the ovarian homogenates was determined as described by Gorban & Boyd (1977), the method being based on the hydrolysis of [¹⁴C]cholesterol ester. The ability of cAMP (10 µM) to activate this hydrolase in vitro was also determined as described by Gorban & Boyd (1977). In these studies, usually only one ovary from each animal was used in the preparation of the 105 000 gₛ supernatant while the other ovary was homogenized in chloroform/methanol for subsequent progesterone determination.

Radioimmunoassays

Progesterone and pregnenolone were measured by the specific RIAs described and validated previously (Neal, Baker, McNatty & Scaramuzzi, 1975; Mason, Arthur & Boyd, 1978a). Progesterone was extracted from serum before assay with petroleum ether. The extraction efficiency was monitored by the addition of [³H]progesterone and the mean recovery was 84%. The progesterone antiserum was prepared against 11α-hydroxyprogesterone hemisuccinate conjugated to bovine serum albumin (BSA). The pregnenolone antiserum was generated against 20-(O-carboxymethyl)-oxime–BSA. Both antisera showed negligible cross-reactivity (<1%) with C₂₇-sterols. The progesterone antiserum cross-reacted <1% with pregnenolone and the pregnenolone antiserum cross-reacted <1% with progesterone. Cross-reactivity with other C₂₁ steroids was <10% for both antisera. The limit of sensitivity of the progesterone and pregnenolone assays was 25 and 50 pg per tube respectively. The intra- and inter-assay variations of both assays were each <13%.

Statistical analysis

The Wilcoxon rank sum test was used to determine whether separate sets of observations differed significantly.

Results

Effect of LH on serum progesterone and ovarian steroid and sterol concentrations

The results in Table 1 show that LH initiated a rapid and sustained increase in serum and ovarian progesterone concentrations although there were marked fluctuations in the extent of the increases during the 72 h period; the greatest increases were at 6, 12 and 48 h after LH. Ovarian pregnenolone concentrations were also increased by LH and remained elevated throughout the 72 h period. There was a slight, but significant (P < 0.05), decrease in ovarian cholesterol...
concentration 6 h after LH administration, but by 12 h the values were similar to those at 0 h and remained at this level for the remainder of the study. The ovarian cholesterol ester concentration was also slightly, but significantly (P < 0.05) reduced 6 h after LH but rose steadily thereafter and by 72 h was double the 0 h value.

Table 1. Effect of LH on serum progesterone and ovarian progesterone, pregnenolone, cholesterol and cholesterol ester concentrations (mean ± s.e.m. for at least 4 rats/group)

<table>
<thead>
<tr>
<th>Time after LH (h)</th>
<th>Serum progesterone (ng/ml)</th>
<th>Ovarian progesterone (ng/mg)</th>
<th>Ovarian pregnenolone (ng/mg)</th>
<th>Ovarian cholesterol (μg/mg)</th>
<th>Ovarian cholesterol ester (μg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.2 ± 0.5*</td>
<td>0.2 ± 0.04*</td>
<td>0.2 ± 0.05*</td>
<td>2.0 ± 0.2*</td>
<td>6.4 ± 0.6*</td>
</tr>
<tr>
<td>6</td>
<td>52 ± 9*</td>
<td>9.0 ± 1.5*</td>
<td>1.4 ± 0.1*</td>
<td>1.2 ± 0.1*</td>
<td>4.1 ± 0.7*</td>
</tr>
<tr>
<td>12</td>
<td>43 ± 7*</td>
<td>6.9 ± 0.4*</td>
<td>1.1 ± 0.1*</td>
<td>1.9 ± 0.1*</td>
<td>4.7 ± 0.9*</td>
</tr>
<tr>
<td>24</td>
<td>6.8 ± 0.8*</td>
<td>3.0 ± 0.1*</td>
<td>0.9 ± 0.09*</td>
<td>2.3 ± 0.1*</td>
<td>9.4 ± 0.8*</td>
</tr>
<tr>
<td>48</td>
<td>34 ± 6*</td>
<td>6.6 ± 0.7*</td>
<td>0.4 ± 0.06*</td>
<td>2.5 ± 0.1*</td>
<td>9.9 ± 1.1*</td>
</tr>
<tr>
<td>72</td>
<td>18 ± 4*</td>
<td>2.8 ± 1.0*</td>
<td>0.5 ± 0.10*</td>
<td>2.7 ± 0.3*</td>
<td>13.3 ± 1.4*</td>
</tr>
</tbody>
</table>

Differences between means with different superscripts in the same column are significant (P < 0.05).

Effect of LH on cholesterol ester hydrolase activity

In Exps I and II, cholesterol ester hydrolase activity was determined at 3 and 6 h after LH treatment (Table 2) when ovarian cholesterol ester concentration falls and ovarian progesterone concentrations are at their highest (Table 1). At both times there was a reduction in the basal activity of the enzyme and its capacity to be activated by cAMP. Similar results were obtained in Exp. III in which the basal activity of the enzyme and its sensitivity to cAMP were still suppressed 48 h after LH treatment (Table 2) when ovarian cholesterol ester concentration was rising and ovarian progesterone concentration was again at its highest (Table 1). In all 3 experiments the inhibitory effects of LH on the hydrolase activity were accompanied by increases in the ovarian progesterone concentrations.

Table 2. Effect of LH on basal and cAMP-activated ovarian cholesterol ester hydrolase activity

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Treatment</th>
<th>Time of autopsy (h)</th>
<th>Cholesterol ester hydrolase activity (pmol oleic acid released/mg protein/min)</th>
<th>Ovarian progesterone (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Saline (5)</td>
<td>3</td>
<td>139 ± 3</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>LH (5)</td>
<td>3</td>
<td>94 ± 7**</td>
<td>13 ± 0.6**</td>
</tr>
<tr>
<td>II</td>
<td>Saline (5)</td>
<td>6</td>
<td>209 ± 12</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>LH (5)</td>
<td>6</td>
<td>169 ± 15*</td>
<td>6.3 ± 1.8**</td>
</tr>
<tr>
<td>III</td>
<td>LH (12)</td>
<td>0</td>
<td>116 ± 12</td>
<td>0.33 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>LH (12)</td>
<td>6</td>
<td>73 ± 10*</td>
<td>3.7 ± 0.7**</td>
</tr>
<tr>
<td></td>
<td>LH (12)</td>
<td>48</td>
<td>65 ± 6**</td>
<td>4.7 ± 0.7**</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. for the no. of replicate determinations indicated in parentheses.
Values significantly different from the corresponding saline (Exps I and II) or 0 h (Exp. III) values: *P < 0.05; **P < 0.01.

Effect of LH on ovarian mitochondrial cytochrome P-450, pregnenolone, progesterone and cholesterol concentrations

The mitochondrial concentrations of pregnenolone and progesterone were elevated 6 h and 48 h after LH (Table 3). Cytochrome P-450 concentrations were unchanged after LH, as were the cholesterol concentrations. The mean mitochondrial cholesterol values, however, followed a pattern similar to that for ovarian cholesterol (Table 1) in that there was a fall after 6 h and a return towards the 0 h values by 48 h.
Table 3. Effect of LH on ovarian mitochondrial cytochrome P-450, pregnenolone, progesterone and cholesterol concentrations (mean ± s.e.m. for 6 replicates)

<table>
<thead>
<tr>
<th></th>
<th>Time after LH administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Cytochrome P-450 (nmol/mg protein)</td>
<td>0.87 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pregnenolone (ng/mg protein)</td>
<td>16 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Progesterone (ng/mg protein)</td>
<td>57 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholesterol (μg/mg protein)</td>
<td>71 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values in the same row with different superscript letters are significantly different (P < 0.05).

Steroid production by ovarian mitochondria in vitro

Ovarian mitochondria were prepared from PMSG-primed rats injected with LH or saline and killed 6 h later. Steroid production in response to endogenous substrate and exogenous cholesterol or 24-hydroxycholesterol was compared (Text-fig. 1). The activity of the cholesterol side-chain cleavage enzyme with endogenous substrate, as estimated by the relative amount of pregnenolone plus progesterone produced during the incubation period was significantly greater (P < 0.01) in mitochondria obtained from the LH treated rats than in those from control rats. Basal pregnenolone production by mitochondria from LH-treated rats fell during the incubation period, perhaps reflecting increased conversion to progesterone, the levels of which increased 2.5-fold between 2 and 20 min of incubation. Addition of cholesterol to mitochondrial

Text-fig. 1. Pregnenolone and progesterone formation in vitro by ovarian mitochondria prepared 6 h after treatment of PMSG-primed immature rats with LH or saline (mean ± s.e.m. for 6 replicates).
incubations from control rats stimulated pregnenolone production by about 20-fold. There was a further 2–3-fold increase ($P < 0.05$) in pregnenolone production when mitochondria from LH treated rats were incubated with cholesterol. Addition of 24-hydroxycholesterol to mitochondria from control rats similarly increased pregnenolone production 20–30-fold relative to basal values, but there was no further increase in pregnenolone production when mitochondria from LH treated rats were incubated with 24-hydroxycholesterol. Similar findings were obtained with 25- and 26-hydroxycholesterol (data not shown).

While pregnenolone production was stimulated 20–30-fold after addition of cholesterol or 24-hydroxycholesterol to mitochondria from control rats, there was no increase in progesterone production by these mitochondria. Only mitochondria from LH treated rats had the capacity to metabolize pregnenolone to progesterone, as indicated by the steady increase in progesterone during the incubation period.

Discussion

Previous studies of the ovary and adrenal (Behrman & Armstrong, 1969; Behrman, Armstrong & Greep, 1970; Boyd, Arthur, Beckett, Mason & Trzeciak, 1975) have demonstrated that acute stimulation of steroidogenesis by LH and ACTH respectively is accompanied by a depletion of cellular cholesterol ester and a concomitant increase in cholesterol ester hydrolyase activity. Together these findings suggest that cellular cholesterol ester may be an important source of the free cholesterol necessary to sustain increased steroidogenesis. There was little evidence for this in the present study, however, which was designed to investigate more chronic stimulation of steroidogenesis. After administration of an ovulatory dose of LH to PMSG-primed immature rats, ovarian pregnenolone and progesterone levels rose rapidly, and although fluctuating, remained elevated, relative to 0 h values, over the entire 72 h period while serum progesterone concentrations mimicked those of the luteal phase in mature cyclic rats (Smith, Freeman & Neill, 1975). While there was a slight, but significant, drop in ovarian cholesterol ester concentration 6 h after LH, the values rose rapidly thereafter and by 72 h were twice the 0 h value. Sustained increased steroidogenesis was therefore associated with an accumulation rather than a depletion of ovarian cholesterol ester, as found by Schuler, Scavo, Kirsch, Flickinger & Strauss (1979) in studies with rats induced to superovulate. The transient decline in cholesterol ester seen 6 h after LH is most probably due to a reduction in synthesis rather than increased hydrolysis since both basal and cAMP-stimulated hydrolyase activity was suppressed during the first 6 h (Table 2). The initial reduction in free cholesterol concentration would also be compatible with cholesterol ester synthesis being inhibited by lack of substrate. The activity of the cholesterol ester hydrolyase was still suppressed 48 h after LH treatment and this sustained, reduced activity may have contributed towards the gradual increase in ovarian cholesterol ester concentration seen between 6 and 72 h after LH. As found in other studies (Bisgaier, Treadwell & Vahouny, 1979), cAMP activated cholesterol ester hydrolyase in vitro. Although LH is thought to mediate its actions through cAMP (Marsh, 1976; Sala et al., 1979) administration of LH in vivo depressed both the basal activity of the hydrolyase and its capacity to be activated by cAMP in vitro (Table 2). The reason for this apparent disparity is unknown, but highlights the fact that the behaviour of an enzyme in vitro may not necessarily reflect its activity in vivo where several factors may be acting to influence its activity. For example, one or more of the proteins/peptides induced by LH (Landfield, Campbell & Midgley, 1979) may also be capable of influencing cholesterol ester hydrolyase activity. These findings do, however, indicate that activation of the hydrolyase and hydrolysis of cholesterol ester is not obligatory in the mechanism by which LH chronically stimulates ovarian steroidogenesis. Moreover, ovarian cholesterol ester is unlikely to be the primary source of cholesterol utilized to maintain the increased steroidogenesis. Studies by Christie, Strauss & Flickinger (1979) indicate that cholesterol derived from plasma lipoproteins is the principal source of cholesterol used to sustain ovarian
progesterone production. Studies on the mechanism of action of ACTH in the adrenal suggest that ACTH may similarly be capable of stimulating steroidogenesis without activating cholesterol ester hydrolase and inducing hydrolysis of cholesterol ester (Pedersen & Brownie, 1979).

Cytochrome P-450 is an essential component of the mitochondrial enzymes involved in cholesterol side-chain cleavage (Simpson, 1979). The finding that LH treatment did not change mitochondrial cytochrome P-450 concentration indicates that the increased steroidogenesis was not a consequence of LH causing an increase in the concentration of cholesterol side-chain cleavage enzyme. Previous studies have suggested that cAMP induced by LH stimulates steroidogenesis through activating a mitochondrial protein kinase which in turn activates the cholesterol side-chain cleavage enzymes (Marsh, 1976; Downing & Dimino, 1979). In the present studies, it was found that addition of cholesterol to mitochondrial incubations from control animals increased pregnenolone formation 20-fold and that this was only increased a further 2–3-fold when mitochondria from LH-treated animals were used (Text-fig. 1). The greatest increase in pregnenolone formation therefore occurs in response to increasing the cholesterol environment of the mitochondria, which suggests that while LH may activate the side-chain cleavage enzymes to some extent, the major limitation to pregnenolone formation is the availability of cholesterol and not the activity of the cleavage enzymes. Steroidogenesis stimulated by LH in vivo was not, however, accompanied by any noticeable increase in mitochondrial cholesterol concentration (Table 3). Indeed, there was a tendency towards a reduction in the cholesterol concentration which might reflect some utilization of the mitochondrial cholesterol for the increased steroidogenesis. Rather than promoting a net increase in mitochondrial cholesterol concentration, LH may act by facilitating the movement of cholesterol to the sites of steroidogenesis within the mitochondria. Evidence for this is provided by the finding that while LH pretreatment stimulated mitochondrial pregnenolone production in vitro in response to exogenous cholesterol, it had no stimulatory effect on pregnenolone formation in response to exogenous 24-hydroxycholesterol (Text-fig. 1). Being more polar, hydroxysterols are likely to traverse mitochondrial membranes with greater ease than cholesterol (Mason et al., 1978b) and so more readily reach the site of side-chain cleavage which is thought to be located in the inner mitochondrial membrane (Yago et al., 1970). Intramitochondrial translocation of cholesterol to the steroidogenic sites in the adrenal is slow and probably rate-limiting, and ACTH may act primarily to facilitate this translocation (Mason et al., 1978a). The results of the present study and those of Toaff, Strauss, Flickinger & Shattil (1979) indicate that a similar situation may occur in the ovary and that the primary action of LH may be to stimulate intramitochondrial movement of cholesterol to the steroidogenic sites. By this mechanism LH does not stimulate pregnenolone formation in response to 24-hydroxycholesterol because the intramitochondrial movement of 24-hydroxycholesterol is not rate-limiting in the steroidogenic process.

While mitochondria from control and LH-treated animals could metabolize exogenous cholesterol and 24-hydroxycholesterol to pregnenolone, it was only mitochondria from LH treated animals that could metabolize this pregnenolone to progesterone (Text-fig. 1). The initiation of luteal progesterone production in response to LH therefore requires not only an increase in pregnenolone formation but is dependent also on LH acting to promote the metabolism of pregnenolone to progesterone.

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References


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