Influence of Luteinizing Hormone and Adenosine 3':5'-Cyclic Monophosphate on the Metabolism of Free and Esterified Cholesterol in Mouse Leydig-Cell Tumours

By WILLIAM R. MOYLE, ROBERT L. JUNGAS and ROY O. GREEP

Laboratory of Human Reproduction and Reproductive Biology and the Department of Biological Chemistry,
Harvard Medical School, Boston, Mass. 02115, U.S.A.

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1. Male C57B1/6J mice bearing Leydig-cell tumours known to synthesize steroids in response to luteinizing hormone (LH) were given intravenous injections of [1,2-³H]cholesterol (50–100 μ Ci per animal). Single-cell suspensions were prepared from the tumours 5–9 days after the injection of [³H]cholesterol and were incubated at 37°C in foetal calf serum supplemented with 50 mM-Tris-HCl, pH 7.4. At various times after the start of incubation cells were collected by filtration of portions of the suspension and their sterols analysed. Within 10 min after LH (5 μ g/ml) or 3':5'-cyclic AMP (20 mM) was added to the cell suspensions an increased conversion of ester cholesterol into free cholesterol could be demonstrated. 2. To observe this rapid effect of LH it was necessary to incubate the cells for 60 min before addition of hormone. 3. The specific radioactivity of testosterone produced was approximately equal to that of the intracellular cholesterol regardless of the presence or absence of LH. 4. The amount of free cholesterol produced in response to LH was far greater than that needed for steroid synthesis. 5. Free cholesterol, but not esterified cholesterol, was released into the incubation medium linearly with time and this release was unaffected by LH. LH may stimulate steroidogenesis in part by increasing the concentration of free cholesterol within the cell.

LH* has been shown to stimulate steroidogenesis in ovarian and testicular tissues of many species *in vitro* (Eik-Nes, 1964). Inasmuch as the stimulation of steroidogenesis could be demonstrated under conditions where no change in the total tissue concentrations of free or esterified cholesterol was observed (Moyle & Armstrong, 1970; Armstrong *et al.*, 1964), the increase in steroidogenesis did not appear to result from an increased supply of cholesterol, the immediate steroidogenic precursor. The administration of LH to female rats, however, does cause a depletion of esterified cholesterol in the ovaries (Herbst, 1967; Armstrong, 1968). Since this effect of LH on cholesterol metabolism *in vivo* was not altered when steroidogenesis was inhibited by aminoglutethimide phosphate (Behrman *et al.*, 1970), the depletion could not have resulted simply from the increased removal of cholesterol for steroid synthesis in the presence of LH. Instead, a more direct effect of LH on cholesterol metabolism was suggested. We have therefore re-examined the effects of LH on cholesterol metabolism in testicular tissue *in vitro*. Tissue derived from Leydig-cell tumours (Moyle & Armstrong, 1970) seemed ideally suited for this purpose, since steroidogenesis in this tissue can be markedly enhanced by LH *in vitro* (Moyle *et al.*, 1971b).

* Abbreviation: LH, luteinizing hormone.

Results presented in an earlier report (Moyle & Armstrong, 1970) showed that LH had little or no effect on the content of free or esterified cholesterol in Leydig-cell tumours during incubation for 1 or 2 h in Krebs-Ringer bicarbonate buffer. Since cholesterol is insoluble in this medium it is probably unable to leave the cell. Thus, were LH to stimulate the hydrolysis of cholesterol esters, the free cholesterol so formed might accumulate inside the cell and inhibit further hydrolysis. For this reason foetal calf serum, which is capable of absorbing cholesterol, was used as the incubation medium in the present experiments. Under these conditions a clear effect of LH on cholesterol ester metabolism could be demonstrated.

A preliminary account of this research has been published (Moyle *et al.*, 1971a).

Experimental

Preparation of tumour-cell suspensions

Leydig-cell tumours were grown in C57B1/6J mice as described by Moyle & Armstrong (1970). The tumour-bearing mice were injected via the tail vein with 50 or 100 μ Ci of [1,2-³H]cholesterol (50 Ci/mmol), obtained from New England Nuclear Corp., Boston, Mass., U.S.A. The sterol was dissolved in 0.1 ml of acetone-propylene glycol (1:1, v/v) and mixed with 1 ml of 0.15 M-NaCl containing 1% bovine

serum albumin (type F; Sigma Chemical Co., St. Louis, Mo., U.S.A.). Each animal received 0.1 ml of the mixture.

At 5–9 days after cholesterol injection the tumours were excised, minced with scissors, and forced through nylon marquisette or Dacron–Ninon into Krebs–Ringer bicarbonate buffer (Umbreit *et al.*, 1957) as described previously (Moyle & Armstrong, 1970). The suspensions so obtained consisted of single cells and small clumps of cells and were centrifuged at 160g for 5 min. The sedimented cells were resuspended in 0.15 M-NaCl and then forced repeatedly in and out of a 10 ml serological pipette. The tip of the pipette was pressed gently against the bottom of the tube as an aid to cell dispersion. Remaining cell clumps were allowed to settle out, after which the suspension was transferred to a second tube and centrifuged at 160g for 10 min. This procedure yielded large numbers of single cells without enzyme treatment.

Procedure for incubation

The cells were resuspended in a medium consisting of foetal calf serum supplemented with 139 mg of Trizma base and 606 mg of Trizma hydrochloride (Sigma) per 100 ml. The resulting pH was 7.4 at 37°C. The calf serum was obtained from Microbiological Associates, Bethesda, Md., U.S.A., and before use was centrifuged at 8000g_{av.} to remove particles that appeared on thawing. Either 0.15 M-NaCl, LH (NIH-LH-S-16, at a final concn. of 5 µg/ml) or cyclic AMP (final concn. 20 mM) was added at various times after the start of the incubation. Portions (1 ml) of medium containing approx. 100 µg of cell protein were removed at various times and the cells were separated from the medium by filtration through cellulose ester filters (Millipore Corp., Bedford, Mass., U.S.A.; pore size 0.3 µm).

Analysis of sterols

The filters containing the cells were added to tubes containing 5 ml of chloroform–methanol (2:1, v/v) and left overnight. After the filters and solvent had been mixed thoroughly, the filters were removed from the solvent with forceps and discarded. Carrier cholesteryl palmitate (250 µg), cholesterol (100 µg), progesterone (25 µg) and testosterone (25 µg) were added to each sample in 0.1 ml of benzene. The lipids were isolated and subjected to t.l.c. as previously described (Moyle & Armstrong, 1970) except that the eluting solvent was hexane–diethyl ether–acetic acid (15:10:1, by vol.). Areas corresponding to the sterols and sterol esters were transferred into vials containing 5 ml of scintillation fluid and counted for radioactivity in a Packard Tricarb scintillation counter. The scintillation fluid consisted of 500 ml

of xylene, 500 ml of dioxan, 120 g of naphthalene, 7.5 g of 2,5-diphenyloxazole and 0.075 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene.

The medium filtrate was dissolved in 10 ml of chloroform–methanol (1:1, v/v) and the lipids were separated from the aqueous phase by addition of 5 ml of chloroform and 2 ml of 0.15 M-NaCl. After centrifugation, the chloroform phase was removed and dried at 50°C under a stream of N₂. The lipids were separated and counted for radioactivity as described for the filters.

In other experiments tumour fragments removed at different times after injection of labelled cholesterol were homogenized in 0.25 M-sucrose–0.001 M-EDTA, pH 7.4, with an all-glass Ten Broeck apparatus. Subcellular fractions were isolated from the homogenate by sequential centrifugation at 1400g_{av.} (10 min), 7000g_{av.} (10 min), 30000g_{av.} (20 min) and 140000g_{av.} (60 min). The lipids were extracted from each fraction with 20 vol. of chloroform–methanol (2:1, v/v) as described above, and free and esterified sterols were separated in the same manner. The esterified cholesterol was saponified in 3 ml of ethanol–60% (w/v) KOH (9:1, v/v) by heating the solution at 85°C for 2 h and the cholesterol was extracted with 2 × 5 ml of hexane. The hexane was evaporated with N₂ and the cholesterol isolated by t.l.c. as described above. Portions of the free and saponified cholesterol were assayed for radioactivity as described above. Cholesterol mass was determined by a modification of the procedure of Zlatkis & Zak (1969). This consisted of dissolving the cholesterol in 1 ml of acetic acid containing 0.5 mg of *o*-phthaldehyde (Sigma) and adding 0.5 ml of conc. H₂SO₄. The *E*₅₄₀ was proportional to the cholesterol content within the range 1–25 µg.

Screening for LH-responsive tumours

The small clumps of tissue remaining after preparation of the cell suspensions were incubated with and without LH (25 µg/ml) in Krebs–Ringer bicarbonate buffer for 1 h. Steroids were extracted and separated by t.l.c. on plates coated with silica gel G containing a fluorescent indicator as described above. Under u.v. light, steroids having the 4-en-3-one structure appeared as dark spots. An effect of LH was evident if the size of the spots in the LH-treated group was considerably larger than those in the control. Only results obtained from tumours responding qualitatively to LH are included in the present paper.

In one experiment a more concentrated cell suspension, containing 2.2 mg of protein/ml, was used to allow the determination of the specific radioactivities of the steroids and sterols isolated. The steroids isolated by t.l.c. as described above were analysed by g.l.c. (Moyle & Armstrong, 1970). The specific radioactivity of the sterols was measured by scintillation

counting of areas isolated by t.l.c.; the mass was determined by a modification of the Lieberman-Burchard reaction (Armstrong *et al.*, 1964).

Results

Specific radioactivity of sterols in tumour subcellular fractions

Table 1 shows the distribution of labelled sterols in subcellular fractions prepared from Leydig-cell tumours at various times after injection of [1,2-³H]-cholesterol. At each time-period the specific radioactivities of free and esterified cholesterol were similar. No differences in specific radioactivity were seen among the subcellular fractions. The specific radioactivity of the isolated sterols was greater in the older, larger tumours.

Effect of LH on concentrations of free and esterified cholesterol

Tumour-cell suspensions were prepared from animals injected with 50 or 100 μ Ci of [1,2-³H]chol-

esterol 5-9 days previously and incubated with or without LH as described above. After 1 h the suspensions were filtered and the residue remaining on the filters was analysed. Table 2 shows the amounts of labelled free and esterified cholesterol recovered. This is assumed to represent sterols which were either within the cells or bound to the cellular surfaces. Cells incubated in the presence of LH yielded less radioactivity in esterified cholesterol and more in free cholesterol. Since the previous experiment demonstrated that the specific radioactivity of these fractions is equal these results indicate that LH has increased the amount of cellular free cholesterol at the expense of esterified cholesterol. Radioactivity isolated in the steroid fractions (progesterone and testosterone) was too small to measure.

The time-course of the LH effect on sterol concentrations is shown in Fig. 1. Quadruplicate groups of cells were incubated in 15 ml of fortified serum for 1 h and 1 ml portions from each group analysed at various intervals. After 1 h of incubation, LH was added and the radioactivity in the sterols was assayed at 5 min intervals for 30 min and less frequently for

Table 1. *Distribution of labelled cholesterol in subcellular fractions of homogenates of Leydig-cell tumours*

Three mice having tumours of approximately equal size were injected with [1,2-³H]cholesterol (50 μ Ci) on day 0. On different days after injection one mouse was killed and the specific radioactivity of the tumour cholesterol determined. Values represent d.p.m./ μ g of cholesterol and are the average of two determinations. n.d., Not determined.

Time after injection (days) ...	Sp. radioactivity (d.p.m./ μ g)					
	1		4		7	
	Free cholesterol	Esterified cholesterol	Free cholesterol	Esterified cholesterol	Free cholesterol	Esterified cholesterol
Fraction						
Pellet (1400g _{av.})	107	53	305	300	491	504
Pellet (7000g _{av.})	n.d.*	n.d.	n.d.	n.d.	495	n.d.
Pellet (140000g _{av.})	n.d.	n.d.	n.d.	n.d.	480	n.d.
Supernatant (140000g _{av.})	n.d.	100	n.d.	335	579	480
Total protein (mg)	19		39		146	

* Not determined because of low cholesterol mass.

Table 2. *Effect of LH on cholesterol metabolism in suspensions of mouse Leydig-cell tumours*

For details see the text. Values are means of single determinations from four flasks and are d.p.m. recovered $\pm$ s.e.m. The effect of LH on free and esterified cholesterol radioactivity is significant at $P < 0.01$ (Student's *t* test).

Expt. no.	Free cholesterol		Cholesterol ester	
	0.85% NaCl	+LH	0.85% NaCl	+LH
1	1295 $\pm$ 24	1640 $\pm$ 20	1846 $\pm$ 26	1479 $\pm$ 17
2	203 $\pm$ 10	260 $\pm$ 17	263 $\pm$ 9	200 $\pm$ 13
3	805 $\pm$ 17	958 $\pm$ 21	774 $\pm$ 20	669 $\pm$ 27

Fig. 1. *Effect of LH on cell cholesterol*

[1,2-³H]Cholesterol (50 μ Ci) was injected intravenously into a tumour-bearing C57B1/6J mouse. Five days after injection the tumour was excised and a cell suspension in foetal calf serum prepared as described in the text. The suspension was incubated in quadruplicate flasks, 1 ml portions being removed from each for analysis at the times indicated on the abscissa. Change in radioactivity: —, cholesterol; ---, esterified cholesterol. LH (5 μ g/ml) was added (vertical arrow) after 1 h of incubation. Vertical bars represent the s.e.m. When LH was not added the rate of loss of both cholesterol and esterified cholesterol continued as during the first hour (results not shown).

Fig. 2. *Time-course of release of cholesterol*

The rate of appearance of cholesterol in the incubation medium, from the experiment described in Fig. 1, is shown. Each point represents the average of portions from four separate flasks. Vertical bars extend to the s.e.m. No labelled esterified cholesterol was observed in the medium.

another 30 min. Within 10 min after addition of LH, radioactivity in free cholesterol of the treated cells exceeded that of the controls. The amount of labelled cholesterol found in the medium was unaffected by LH in these experiments, as shown in Fig. 2. Almost no radioactivity was detected in the cholesterol ester fraction of the serum. Either very little esterified cholesterol was able to leave the cell before being hydrolysed or esters appearing in the medium were hydrolysed rapidly.

Effect of LH on steroidogenesis

Table 3 summarizes the results of an experiment with larger quantities of tumour cells to allow measurement of the effect of LH on the mass of steroids present. LH increased testosterone synthesis severalfold, but the specific radioactivity of the steroid was unaffected. This failure to detect a significant decrease in the specific radioactivity of the testosterone produced after LH stimulation, in spite of the large quantities of unlabelled cholesterol present in the serum medium, indicates that serum cholesterol is not an important source of steroid precursor during the immediate response to LH.

Table 3. *Effect of LH on sterol and steroid specific radioactivity*

Tumour cells were incubated in foetal calf serum as described in the Experimental section. After 1 h the incubation was terminated and the mass and radioactivity of the free and esterified cholesterol and of testosterone were determined. The specific radioactivity of the free and esterified cholesterol was determined for the serum plus cells, except for values in the bottom row which represent the specific radioactivity of the sterols in the intact cells before addition to serum. Values represent the means of duplicate measurements on the contents of one flask at each point.

Addition	Free cholesterol (d.p.m./ μ mol)	Cholesterol ester (d.p.m./ μ mol)	Testosterone	
			(d.p.m./ μ mol)	(μ g)
0.85% NaCl	27.0	7.7	100.8	0.01
LH (50 μ g/ml)	31.2	6.6	87.3	0.11
Before addition to serum	106.5	99.6	—	—

Table 4. *Effect of 3':5'-cyclic AMP on cholesterol metabolism*

Tumour cells were incubated in foetal calf serum containing 0.01 M-theophylline for 30 min. After this 0.85% NaCl (control) or 3':5'-cyclic AMP (final concn. 0.02 M) was added to the cells and incubation continued for another 30 min. Values represent the average percentage of radioactivity found in each fraction $\pm$ S.E.M. for four flasks.

Incubation conditions	Average radioactivity (% of total d.p.m.)		
	Cellular free cholesterol	Cellular esterified cholesterol	Medium cholesterol
30 min only	50.0 $\pm$ 1.4	46.5 $\pm$ 1.2	3.8 $\pm$ 0.3*
60 min (control)	45.8 $\pm$ 0.5*	44.0 $\pm$ 1.1	10.3 $\pm$ 0.7
60 min + cyclic AMP	49.8 $\pm$ 0.2	37.8 $\pm$ 0.5**	10.8 $\pm$ 0.5

* Significantly different from other values in the same column at $P < 0.001$ (t test).

** Significantly different from other values in the same column at $P < 0.025$ (t test).

Moreover, the specific radioactivity of the free and esterified cholesterol within the cell (measured on cells not incubated in serum) was approximately equal to that of the testosterone synthesized during the incubation period.

Effect of cyclic AMP on cholesterol metabolism of tumour cells

Addition of cyclic AMP to tumour cells suspended in foetal calf serum promoted the same changes in cholesterol metabolism as seen with LH and they are shown in Table 4. Cyclic AMP has been implicated as the mediator of LH action on steroidogenesis (Marsh, 1970; Marsh & Savard, 1966) and the observation that cyclic AMP increased the concentration of free cholesterol at the expense of ester cholesterol is consistent with this interpretation.

Discussion

The present results indicate that LH promotes the conversion of ester cholesterol into free cholesterol

in Leydig cells with little if any lag period. Since esterified cholesterol is not a substrate for the cholesterol side-chain-cleaving enzyme in the tumour cells (Moyle *et al.*, 1970), it is clear that one of the actions of LH is to increase the availability of free cholesterol, the substrate for steroidogenesis. Because only a small fraction of the cholesterol in the cells is converted into steroids and because Behrman *et al.* (1970) have shown that aminogluthethimide phosphate, an inhibitor of steroidogenesis, does not inhibit the cholesterol ester-depleting effect of LH in the ovary, it seems unlikely that the effect of LH on cholesterol ester concentrations observed here is secondary to its effects on steroid synthesis. Although it is possible that the increased side-chain cleavage seen in the presence of LH (Hall & Young, 1968) results from an increased availability of cholesterol to the enzyme system, a separate and additional effect of LH on the cleavage enzyme cannot be ruled out. There is some evidence, however, that a supply of cholesterol ester must be available if a response to LH is to be obtained (Cook *et al.*, 1967). Indeed, it has been our experience that certain of the Leydig-cell tumours fail to respond

to LH and these tumours characteristically have a very low content of cholesterol ester, whereas free cholesterol concentrations are the same in responsive and unresponsive tumours.

The effect of LH on cholesterol metabolism in rat ovaries apparently results from a stimulation of the activity of the sterol ester hydrolase (EC 3.1.1.13) in response to LH (Behrman & Armstrong, 1969). As cyclic AMP mimicked the effect of LH on the conversion of cholesterol ester into free cholesterol in the tumour cells, cyclic AMP may be mediating the LH effect by activating the hydrolase in the tumour cells. Recent studies of the adipose tissue triglyceride lipase indicate that cyclic AMP increases lipolysis by activating a protein kinase, which phosphorylates and thus activates the lipase (Corbin *et al.*, 1970; Huttunen *et al.*, 1970a,b). As the ester hydrolase and the triglyceride lipase catalyse analogous reactions and appear to be stimulated by cyclic AMP-mediated processes, it will be of interest to determine whether the stimulation of the hydrolase also involves a cyclic AMP-activated protein kinase.

The time-lag involved in the effect of LH on cholesterol ester hydrolysis depends greatly on the conditions of the experiment. Thus we were able to demonstrate a significant effect within 10 min on tumour cells that had been incubated 1 h before addition of the LH, whereas 20–30 min was required to see this effect when the prior step was omitted. Working *in vivo* with rat ovarian tissue, Armstrong (1968) could not detect this effect until several hours after LH administration, even though LH increased the ovarian steroid content within 30 min. The reasons for this variable delay in detecting an effect of LH on cholesterol metabolism are not clear, but several factors may be considered. The vast bulk of cellular cholesterol is present in lipid droplets and cellular membranes and not in the soluble fraction obtained after isolation of cellular organelles by the technique of differential sedimentation. This soluble cholesterol fraction might represent the 'metabolically active' cholesterol, which is increased by LH treatment. Such a 'metabolically active' cholesterol pool would have to be considerably enlarged before the change could be detected from measurements of total cellular cholesterol. Moreover, substantial expansion of the active cholesterol pool may be difficult to achieve if cholesterol should inhibit the sterol hydrolase in the way fatty acids inhibit the triglyceride lipase in adipose tissue (Jungas, 1970). Therefore a rapid LH effect on cholesterol metabolism can be most readily observed when the 'less-active' pool of cholesterol is first depleted by incubation in a medium able to accept cholesterol from the cells. In other circumstances, even though the action of LH may be initiated very early, the quantitative changes may remain too small to detect reliably for several hours because of the large mass of cholesterol present.

Our previous failure to observe an effect of LH on cholesterol ester conversion into free cholesterol in Leydig-cell tumours incubated in Krebs–Ringer bicarbonate buffer (Moyle & Armstrong, 1970) may be similarly explained. Free cholesterol concentrations in the cells were found to be about 20 µg/mg of protein whereas the total amount of steroids synthesized after stimulation by LH was only 0.5 µg/h per mg of protein. Thus a change of less than 5% in the cholesterol concentration would be necessary to support the increased steroidogenesis measured over the course of 1 h and a change of this magnitude would be very difficult to detect unless the cholesterol content of the cells were depleted by incubating the cells in serum.

The observation that cholesterol already present within the cell serves as the major source of steroids is in agreement with similar conclusions by others (Armstrong, 1967; Solod *et al.*, 1966). The fact that cholesterol from the foetal calf serum was not readily converted into steroids indicates that it probably enters the cells slowly. Thus serum cholesterol may be essentially unavailable for the rapid burst of steroidogenesis after treatment with LH.

The finding that cholesterol ester was apparently not released into the serum along with free cholesterol may reflect the general immobility of these esters (Moyle *et al.*, 1970). Thus the esters may be regarded as a storage form of cholesterol within the cell, which feeds into the active pool of free cholesterol. If LH increases steroidogenesis in part by increasing the concentration of 'mobile' free cholesterol within the cell, it is puzzling why no appreciable increase in the rate of release of free cholesterol into the medium was seen.

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