Aust. J. Biol. Sci., 1978, **31**, 405–13

Inhibition of Cholesterol Biosynthesis in Ovine Ovarian Follicles *in vitro* by Human Chorionic Gonadotrophin

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Abstract

Cholesterol biosynthesis from DL-[2-^{14}C]mevalonic acid ([^{14}C]MVA) was demonstrated in ovine ovarian follicles and isolated thecal tissues and granulosal cells incubated *in vitro*. Thecal tissues more readily synthesized cholesterol than did granulosal cells when incubated separately, but in the intact follicle the newly synthesized cholesterol distributed evenly between the two tissue layers, indicating that the theca could act as a supplementary source of cholesterol for the granulosal cells.

Human chorionic gonadotrophin (hCG) added to the incubation medium was found to inhibit cholesterol biosynthesis from [^{14}C]MVA by intact follicles and isolated thecal tissues, but not granulosal cells. This hCG-induced inhibition was evident in whole follicles incubated for 12–48 h, but not at 3–6 h, and was demonstrated in thecal tissues incubated for 3 h. In all cases where inhibition of cholesterol biosynthesis was observed, ^{14}C label accumulated in a product characterized by thin layer and vapour phase chromatography as lanosterol, implying that the hCG block lies between lanosterol and cholesterol.

Treatment of follicles with hCG also reduced the amount of ^{14}C label incorporated into the cholesteryl ester fraction. These changes were accompanied by a corresponding reduction in the tissue content of cholesteryl ester, but there were no changes in the specific activities to indicate that newly synthesized cholesteryl ester was used selectively as a substrate for progestin biosynthesis.

Introduction

Luteinizing hormone (LH) has clearly been shown to stimulate progesterone biosynthesis *in vitro* by ovarian tissues of several species (Savard *et al.* 1965; Armstrong 1968; McIntosh and Moor 1973; Younglai 1977). The stimulatory effect of LH on steroidogenesis appears to be primarily located at post-cholesterol sites in the pathway, at least in rat lutein tissue (Major *et al.* 1967; Solod *et al.* 1967), rabbit ovarian tissue (Armstrong 1967), and bovine luteal tissue (Armstrong *et al.* 1970).

The question as to whether LH also acts to influence cholesterol biosynthesis remains unresolved as Savard (1967) found evidence that incorporation of [1-^{14}C]-acetate into cholesterol in bovine luteal tissues was stimulated by LH, whereas Armstrong *et al.* (1970) presented data, derived from similar studies, which indicate that LH inhibits cholesterol synthesis.

By contrast, during the course of our biochemical investigations of the effect of human chorionic gonadotrophin (hCG) on sheep ovarian follicles in culture, we were unable to demonstrate any changes in the cholesterol content of follicles, concomitant with the marked rise in progestin secretion, induced by this LH-like hormone (Seamark *et al.* 1976*b*). Neither were there any hCG-induced changes

in the cholesteryl ester fraction which is of particular interest as Armstrong *et al.* (1970) had indicted this fraction as the major ovarian source of the cholesterol for progesterone synthesis.

Because of these apparently conflicting data we have examined the effect of hCG on cholesterol biosynthesis by ovine ovarian follicles *in vitro*, using isotope incorporation procedures with DL-[2-^{14}C]mevalonic acid ([^{14}C]MVA) as the precursor.

Materials and Methods

Experimental Material

Ovaries were obtained from sheep of mixed breed (mainly Merino crossbreds) within 40 min of slaughter at the metropolitan abattoir (SAMCOR) and transported to the laboratory in ice-chilled Dulbecco phosphate-buffered saline (Commonwealth Serum Laboratories, Parkville, Vic.) containing 50 μg Kanamycin ml^{-1} (Sigma Chemical Co., St Louis, Missouri, U.S.A.). Follicles between 4 and 6 mm in diameter were dissected from those ovaries containing a corpus luteum; these follicles were morphologically staged as being between days 4 and 14 of the cycle, and they were prepared for culture as described by Moor (1973).

Preparation of Tissues

Where required thecal and granulosal tissues were prepared by microdissection of follicles in Dulbecco phosphate buffer containing calf serum, 4% (w/v) Kanamycin sulphate and EDTA (4 mM) to prevent clotting of the follicular fluid. Each follicle was cut, almost in half, with fine scissors and the granulosal cells removed by gently scraping with a fine glass crook. The granulosal cells obtained were collected by centrifugation (400 rev/min for 5 min), and cells resuspended in HEPES-buffered Medium 199 (Commonwealth Serum Laboratories), and recentrifuged. The washed cells were then resuspended in HEPES-buffered Medium 199 for incubation. The thecal casings were cut into several pieces and incubated in HEPES-buffered Medium 199.

Incubation Procedures

After their dimensions had been recorded the follicles were cultured as described by Moor (1973). The incubation medium employed was HEPES-buffered Medium 199 containing Kanamycin sulphate (1 mg ml^{-1}), foetal calf serum (20%), and insulin (56 mg l^{-1}), pH 7·0, to which was added the specific sterol biosynthesis precursor [^{14}C]MVA (1·25 μCi ml^{-1}; Radiochemical Centre, England) and, where indicated, the appropriate amount of hCG (20 i.u. ml^{-1}; Organon Labs Ltd, Morden, England), and Amo-1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperdine carboxylate methyl chloride; Calbiochem, U.S.A.). The medium with additions was sterilized by Millipore filtration. This concentration of hCG was considered as appropriate because it has been demonstrated to transform the ovine ovarian follicle from an eostrogen to a progesterone producer in culture (McIntosh and Moor 1973). Incubation was carried out at 37°C in the presence of a special gas mixture (5% CO_2, 50% O_2, 45% N_2).

Thecal and granulosal cells were resuspended in HEPES-buffered Medium 199 (as above; 0·5 ml) containing [^{14}C]MVA (5 μCi ml^{-1}) and, where required, hCG (20 i.u. ml^{-1}) and cultured in plastic tubes fitted with special caps to allow circulation of gas. Incubations were carried out with agitation at 37°C.

Extraction, Isolation and Identification of Products

After incubation, follicles were removed from the culture dishes, blotted on to filter paper to remove any excess medium, and homogenized in chloroform–methanol (2 : 1, v/v; 1 ml) in an all-glass homogenizer (Kontes Glass Co., N.J., U.S.A.). Thecal tissues were treated exactly as the intact follicles, whereas granulosal cells were pelletted by centrifugation (400 rev/min for 5 min) in the homogenizer mortar, the medium aspirated off, and then they were homogenized (as above). Homogenates were filtered through glass-wool-plugged Pasteur pipettes to remove lipid-insoluble material and the homogenizer and residues were washed with successive amounts of chloroform–methanol (2 : 1, v/v; 2×2 ml, 2×1 ml). The combined extracts were vortexed with 20% of their volume of a solution of 0·05% (w/v) $CaCl_2$ and, after complete phase separation, the upper (aqueous phase) was aspirated off and the phase interface carefully washed with Folch upper phase (Folch *et al.* 1957). The washed extract was then evaporated to dryness (60°C) under a stream of

nitrogen and the residue redissolved in chloroform–methanol (2 : 1, v/v; $3 \times 100\ \mu l$) and spotted onto silica gel G (E. Merck, Darmstadt, Germany) thin layer plates (0·25 mm by 3 cm by 15 cm) which were developed twice in 4% (v/v) diethylether in dichloromethane. After chromatography, the plates were lightly sprayed with 0·05% (w/v) berberine hydrochloride (Fluka Chemical Co., Bucks, Switzerland) in methanol and reference compounds located by fluorescence under u.v. light. Radioactive metabolites were located using a radiochromatogram scanner (Panax, Surrey, England) and recovered by eluting the silica gel, scraped from the appropriate area of the t.l.c. plate, with diethylether. As cholesteryl ester and squalene-2,3-epoxide cochromatographed in this solvent system, material eluted from this region of the t.l.c. plate was saponified with 30% (w/v) ethanolic KOH (1 ml) for 1 h at 60°C to de-esterify the cholesteryl ester. The mixture was then extracted with light petroleum (boiling range 60–80°C; 3×2 ml) and, after removal of solvent, the residue rechromatographed as above. Squalene-2,3-epoxide and the cholesterol derived from the cholesteryl ester were then obtained by elution.

Gas–liquid chromatography (g.l.c.) was performed on a Pye Unicam (104 series) gas chromatograph fitted with glass columns (1·84 m by 0·4 cm) containing 3% (w/w) OV-17 on Gas Chrom Q (100–120 mesh) (Applied Science Labs, State College, Pa., U.S.A.). The end of the column was fittted with a 1 : 1 stream splitter which connected to a flame ionization detector and a fraction collector (Model 850, Packard Inst. Co., Illinois, U.S.A.). Fractions of eluant were collected into tubes packed with anthracene crystals coated with OV-17 which were counted directly for radioactivity. Operating temperatures were: column 230°C, injection port 220°C, detector 300°C, and collector 250°C. Carrier gas (N_2) flow rates were 60 ml min^{-1}.

Trimethylsilyl (TMS) ethers of cholesterol and lanosterol were prepared by addition of 50 μl of BSTFA (*N,O*-bis(trimethylsilyl)trifluoroacetamide; Pierce, Illinois, U.S.A.) to a solution of 500 μg of the sterol in acetonitrile (50 μl) and heating the mixture (60°C, 30 min). After reaction the solvent was removed (at 60°C under N_2) and the derivative redissolved in light petroleum (boiling range 60–80°C; 100 μl) ready for g.l.c. analysis. For all analyses cholestane (Steraloids, New Hampshire, U.S.A.) was used as internal standard.

Radioactivity was determined with an Isocap/300 liquid scintillation counter (Nuclear Chicago, U.S.A.).

Results

Effect of hCG on Cholesterol Synthesis by Intact Follicles in vitro

Chromatograms carrying extracts obtained from follicles incubated with [^{14}C]MVA for periods of 3–48 h revealed incorporation of ^{14}C label into at least five fractions which cochromatographed with authentic cholesterol, 4-methyl cholesterol, lanosterol, cholesteryl ester–squalene-2,3-epoxide, and squalene. These same compounds were also formed by follicles cultured in the presence of hCG (20 i.u. ml^{-1}), but when compared with untreated follicles there was, after 6 h, a significant decrease in the percentage of recovered radioactivity found in the cholesterol fraction accompanied by an increase in that associated with the presumptive lanosterol, and to a lesser extent the 4-methyl cholesterol and squalene fractions (Table 1). With increasing incubation time this gonadotrophin effect on cholesterol synthesis became more pronounced (Table 1).

Interestingly, no radioactivity was found to be associated with several steroid fractions, including oestradiol and progesterone, known to be actively synthesized by the follicle in culture.

Characterization of Cholesterol and of the Major Accumulation Product following hCG Treatment

(i) *Inhibitor studies*

Amo-1618 has been shown to inhibit cholesterol biosynthesis in ovine ovarian follicles in culture, resulting in an accumulation of label into squalene-2,3-epoxide

Table 1. Effect of hCG on cholesterol biosynthesis in ovine ovarian follicles in culture

Sheep ovarian follicles were incubated with [^{14}C]MVA (1·25 μCi ml^{-1}) for the time period shown and the ^{14}C-labelled products were isolated by chromatography. Where indicated, hCG (20 i.u. ml^{-1}) was included in the incubation mixture (see text for further details). Values shown are mean percentages ± s.d. for three replicates per treatment except where indicated otherwise

Incubation time (h)	Treatment	Total counts incorporated (cpm)	Percentage distribution of ^{14}C label				
			Squalene	Lanosterol	'4-Methyl cholesterol'	Cholesterol	Cholesteryl ester
3	None	1020±181	25·2±4·8	19·7±4·8	7·3±1·6	43·3±9·8	4·5±1·2
	+hCG	849±22	23·4±5·6	22·0±3·7	12·2±5·5	39·1±7·2	3·3±1·1
6	None	2047±114	19·4±5·4	17·5±3·1	7·3±1·6	52·6±11·1	3·2±0·6
	+hCG	2447±204	26·2±5·6	21·0±5·3	17·0±8·2	31·3±6·0	4·5±1·1
12	None	6603±1012	28·6±8·9	19·5±1·9	9·2±0·7	41·3±5·9	1·3±0·1
	+hCG	7640±724	30·1±4·7	31·5±2·8*	13·1±3·7	23·1±4·5	2·2±0·5
19	None[A]	8311±1592	24·2±8·2	9·7±2·7	7·7±1·3	57·3±6·2	1·1±0·2
	+hCG[A]	8781±2328	38·3±9·5	25·5±1·9*	12·1±3·2	38·2±5·3	1·9±0·6
24	None[A]	12187±1852	32·5±4·9	7·5±1·3	3·8±0·6	53·0±3·8	3·2±0·9
	+hCG[A]	14086±2071	38·3±4·5	25·0±2·8*	10·1±1·7*	23·4±3·8*	3·1±0·2
48	None	36537±660	24·1±7·4	11·1±4·7	6·2±1·6	54·4±5·5	4·1±0·7
	+hCG	50143±7302	21·6±6·1	47·0±7·2*	13·4±1·9	14·9±5·8*	3·1±0·3

[A] Four replicates per treatment.

* Statistically significant difference relative to untreated follicles ($P < 0·05$).

(Douglas and Hamilton 1978). When hCG (20 i.u. ml^{-1}) was added to follicles in the presence of Amo-1618 (100 $\mu g\ ml^{-1}$) there was no change in the recovery of radioactivity in the presumptive lanosterol fraction (Table 2), indicating that this hCG-induced accumulation product was formed after squalene-2,3-epoxide in the cholesterol biosynthetic pathway.

Table 2. Effect of hCG with Amo-1618 on cholesterol biosynthesis in ovine ovarian follicles in culture

Follicles (six per treatment) were incubated with [^{14}C]MVA (1·25 $\mu Ci\ ml^{-1}$) for 19 h and the ^{14}C-labelled products were isolated by chromatography. Where indicated, hCG (20 i.u. ml^{-1}) and/or Amo-1618 (100 $\mu g\ ml^{-1}$) were added to the incubation mixture (see text for further details). Values shown are means ± s.d., expressed as counts per minute per milligram wet weight

Treatment	Incorporation of label into:			
	Cholesterol	Lanosterol	Squalene-2,3-epoxide	Squalene
Control	1223·9±523·8	316·0±116·4	44·3±32·3	522·9±200·7
hCG	428·0±237·8	1256·3±549·9	26·7±10·2	482·9±392·6
Amo-1618	322·0±74·7	107·1±75·1	288·7±78·1	268·3±135·2
hCG+Amo-1618	441·0±182·2	247·5±147·1	271·0±130·1	197·1±100·0

(ii) *G.l.c. studies*

The ^{14}C-labelled materials in cholesterol and lanosterol fractions were eluted from chromatograms and mixed with the appropriate authentic sterol for examination by g.l.c. either directly or as the TMS ethers. With the cholesterol fraction all (>95%) of the ^{14}C-labelled substances cochromatographed with authentic cholesterol as the derivative. With the lanosterol fraction in the underivatized sample 69·2% of the total radioactivity cochromatographed with lanosterol, and in the derivatized sample 67·6% of label cochromatographed with lanosterol-TMS.

Effect of hCG on Cholesterol Synthesis by Isolated Thecal and Granulosal Tissues

To ascertain the relative contributions of thecal and granulosal tissues to follicular cholesterol biosynthesis, and further investigate the effects of hCG, follicles were separated into thecal casings and granulosal cells either before or after incubation with [^{14}C]MVA. It was found that the thecal tissues were considerably more active in cholesterol synthesis than granulosal cells when incubated separately, and were also more sensitive to the inhibitory effect of hCG (Table 3). In all four experiments where thecal tissues were incubated separately, hCG (20 i.u. ml^{-1}) added to the medium resulted in an inhibition in the uptake of label into cholesterol (30·4, 34·4, 29·5 and 44·9% in the four experiments) and a concomitant increase of label in the lanosterol fraction (35·0, 31·3, 15·7 and 30·6% respectively). No significant hCG effect was observed in any of the granulosal cell treatments. However, when the follicular tissues were isolated from intact follicles after overnight incubation with [^{14}C]MVA, cholesterol and the other ^{14}C-labelled metabolites were evenly distributed between the two tissue layers which masked any tissue differences in the gonadotrophin effect.

Table 3. Effect of hCG on cholesterol biosynthesis by isolated thecal and granulosal tissues from ovine ovarian follicles

The thecal and granulosal tissues were isolated from intact follicles (*a*) prior to incubation for 3 h with [^{14}C]MVA, and (*b*) after incubation for 17 h with [^{14}C]MVA. Where indicated, hCG (20 i.u. ml^{-1}) was added to the incubation mixture (see text for further details). Values shown are means for four replicates per treatment

Tissue	Treatment	Total counts incorporated (cpm per mg tissue)	Percentage distribution of ^{14}C label				
			Squalene	Lanosterol	'4-Methyl cholesterol'	Cholesterol	Cholesteryl ester
			(*a*) Before incubation				
Theca	Control	1317·4	46·1	24·3	11·5	11·3	3·5
	+hCG	1298·6	53·0	32·2*	8·7	7·7*	3·1
Granulosa	Control	137·0	11·6	10·8	13·9	56·4	3·1
	+hCG	113·5	9·7	10·0	16·0	56·8	3·0
			(*b*) After incubation				
Theca	Control	509·3	11·5	6·9	8·8	71·7	1·2
	+hCG	540·2	3·5	29·3	22·3	44·5	0·4
Granulosa	Control	174·9	3·1	11·7	4·1	80·1	0·3
	+hCG	212·8	1·2	35·8	16·5	16·5	0·6

* Significantly different from control ($P < 0·02$) by *t*-test (paired values).

hCG Effect on Cholesteryl Ester Metabolism

The data presented in Table 1 indicate that prolonged exposure (48 h) of follicles to hCG (20 i.u. ml^{-1}) resulted in a slight reduction in the relative amounts of label incorporated into the cholesteryl ester fraction. To further resolve this effect, the experiment was repeated and a more detailed examination of specific incorporation of [^{14}C]MVA label into the cholesteryl ester fraction was undertaken. Results from two such experiments are presented in Table 4. In both cases the reduction in ^{14}C label incorporated was associated with a corresponding reduction in the tissue content of cholesteryl ester and there was no change in the specific activity of the isolated cholesterol.

Table 4. Effect of hCG on cholesteryl ester levels and specific activity of cholesterol in cholesteryl ester after 48 h incubation of ovine ovarian follicles in the presence of [^{14}C]MVA *in vitro*

Where indicated, hCG (20 i.u. ml^{-1}) was included in the incubation mixture (see text for further details). Values shown are means ± s.d.

Expt No.	Treatment	Cholesteryl ester		
		^{14}C incorporated (cpm)	Tissue content (nmol per follicle)	Specific activity (cpm $mmol^{-1}$)
I	Control	1451·0±145·2	15·4±2·6	96·5±21·2
(n = 3)	+hCG	1266·0±82·4	10·6±1·8	121·5±25·1
II	Control	1112·8±224·0	20·0±4·0	56·1±7·1
(n = 4)	+hCG	528·0±159·7	10·0±0·3	50·7±3·7

Discussion

The data demonstrate that sheep ovarian follicles can synthesize cholesterol from mevalonic acid precursor *in vitro* and that this synthesis mainly occurs in the theca, as isolates of this tissue formed considerably more cholesterol than those of granulosal cells. The data also clearly show that this function is inhibited in both the follicles and isolated thecal tissues by hCG. The site of the hCG-induced inhibition is probably subsequent to the formation of lanosterol in the biosynthetic pathway. This reduction is based on the finding that, in hCG-treated follicles, coincident with the decline in cholesterol formation there is an accumulation of a major product which cochromatographs in both g.l.c. and t.l.c. systems with lanosterol. Furthermore, formation of this product is blocked if the follicle is simultaneously treated with Amo-1618, a powerful inhibitor of cholesterol biosynthesis (Paleg and Seamark 1968; Paleg 1970*a*, 1970*b*) which acts on the cyclization of squalene-2,3-epoxide (Douglas and Paleg 1972), i.e. immediately prior to lanosterol formation.

In thecal tissues the hCG effect on cholesterol biosynthesis was demonstrable within 3 h of treatment but not until 6–12 h with the intact follicle. This suggests that either there is a slower uptake of the gonadotrophin by the intact follicle or that a time interval was required to allow accumulation of some substances involved in mediating the hCG effect.

Treatment with hCG induces numerous biochemical changes in the sheep follicle (Moor *et al.* 1975; Seamark *et al.* 1976*a*), including the formation of several substances which could act on cholesterol biosynthesis. The data of Gaylor and Lee

(1966), for example, suggest that progesterone which is formed in response to hCG could act to reduce cholesterol synthesis by inhibiting the demethylation of lanosterol. However, our preliminary data from experiments with follicles treated with progestins indicate that while inhibition of cholesterol synthesis could occasionally be induced, the effect is nowhere near as consistent as that repeatedly achieved by hCG treatment (data not shown).

In its response to hCG the cholesterol biosynthetic functions of sheep ovarian follicles appear to behave in a similar manner to those found in bovine and rat corpora luteal tissue (Armstrong *et al.* 1969, 1970) in that exposure to LH produced an inhibition of incorporation of ^{14}C-labelled precursor into cholesterol. It seems probable that the reduced cholesteryl ester levels, observed after 48 h incubation of follicles with hCG, are the result of this inhibition of cholesterol synthesis, although inhibition of cholesteryl ester synthesis, as demonstrated by Flint *et al.* (1973) in rat ovarian tissue, could also have contributed to the decreased cholesteryl ester levels. However, in the present studies no evidence was obtained to suggest a preference for steroid synthesis to proceed via the most recently synthesized cholesterol in the sheep follicle *in vitro*, as has been inferred from previous studies with rat ovaries (Flint and Armstrong 1971). Rather, the lack of change in the specific activity of cholesteryl ester following hCG treatment coupled with the observation that negligible amounts of ^{14}C label were associated with several steroid fractions suggest that there is a preferential utilization of the preformed (stored) cholesterol for progesterone production.

The biological implictions of the hCG-(LH)-induced inhibition of cholesterol synthesis remains to be determined. One possible sequel is in its effects on thecal steroid production, as this virtually ceases in the pre-ovulatory follicle after exposure to LH (see Moor *et al.* 1975). These effects on the thecal cell are, however, accompanied by luteinization and the increased progestin production by the membrana granulosa. Furthermore, the poor cholesterol biosynthetic capacity of granulosal cells compared with thecal cells, together with the lack of response of the isolated granulosal tissue to hCG treatment indicate that the granulosal cells may be dependent upon a supply of cholesterol from thecal tissues as a precursor for the synthesis of progestins.

Acknowledgments

The authors gratefully acknowledge the skilled technical assistance of Mrs Regan Jeffrey and Mr Fred Amato.

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Manuscript received 6 December 1977