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Oxygen Uptake, Glucose Utilization, Lactate Release and Adenine Nucleotide Content of Sheep Ovarian Follicles in Culture: Effect of Human Chorionic Gonadotrophin

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Abstract

A study has been made of the oxygen uptake, glucose utilization, lactate release and cellular content of adenine nucleotides of isolated sheep ovarian follicles (4–6 mm in diameter) maintained in organ culture, and of the effects on these parameters of the addition of human chorionic gonadotrophin (hCG).

The mean oxygen consumption of the entire follicles when freshly isolated and of the theca and membrana components was 0·56, 1·08 and 0·05 μmol per milligram wet weight of tissue per hour respectively.

About 8 μmol of glucose was taken up and 16 μmol of lactate released per milligram wet weight of follicular tissue per hour during the first 24-h period of culture. This rate reduced by about 30% for each subsequent day of culture, but was significantly increased by the addition of hCG.

The mean ATP content of theca and granulosa tissues was 4·6 and 2·8 nmol/mg wet weight of tissue respectively. There was no discernable change in tissue adenine nucleotide content or energy charge ratio during the 3-day culture period, and prolonged exposure to hCG was without effect.

Untreated follicles produced both oestrogen and androgens throughout the culture period. The addition of hCG resulted in a transitory stimulation in oestrogen secretion, inhibition of androgen secretion, and a marked and sustained rise in progestin secretion.

It is proposed that the increase in glycolytic activity following exposure to hCG may relate to the activation of the granulosa cells coincident with a transference of steroid synthetic capacity from theca interna to membrana granulosa.

Introduction

Interpretation of results from many biochemical studies of ovarian function are often limited, as whole ovaries rather than individual ovarian tissues have been used. With the recent development of techniques for isolation and maintenance of individual ovarian tissues in culture, more detailed investigation has become possible. Our studies on sheep ovarian follicles have shown that they can be maintained for several days in culture and retain their morphological integrity, endocrine function and capacity to respond to gonadotrophin and other substances (Moor 1973; Seamark *et al.* 1974; Moor *et al.* 1975). More recently, we have reported the feasibility of separating theca and granulosa cells for individual study (Weiss *et al.* 1976).

In this paper we report on studies on the oxygen uptake, glucose uptake, lactate release and cellular content of adenine nucleotides in sheep follicles under conditions of culture and in relation to changes in steroidogenesis induced by human chorionic gonadotrophin (hCG).

Materials and Methods

Ovaries were obtained from mixed breed sheep (Merino cross) within 40 min of slaughter at a local abbatoir and were transported to the laboratory in ice-chilled Dulbecco's Phosphate Buffered Saline containing 50 μg Kanamycin/ml (Sigma, St. Louis, Missouri, U.S.A.).

Follicles were dissected from ovaries containing a corpus luteum morphologically staged at between days 4 and 14 of the oestrous cycle, and established individually in organ culture as described by Moor (1973). Unless otherwise indicated, only medium-sized follicles of 4–6 mm in diameter were taken; on average two such follicles were obtained from each ovary. To separate theca and granulosa tissues, the theca shell was pierced and the granulosa cells extravasated as described previously (Weiss *et al.* 1976). Human chorionic gonadotrophin was obtained from Organon Laboratories Ltd, Morden, Surrey, U.K.

Oxygen uptake of tissues was determined using an oxygen electrode (Rank Bros, Bottisham, Cambs.). The follicle or separated tissues were transferred to the electrode chamber, volume 2 ml, and the respiration determined in culture media. This media comprised media 199 [Commonwealth Serum Laboratories (CSL), Melbourne, Australia], to which was added 16% foetal calf serum (CSL), 47 ng insulin (CSL)/ml, plus 8·3 μg Kanamycin/ml.

The media employed was previously equilibrated in the same atmosphere as used for tissue culture, namely 43% N_2, 5·28% CO_2 and 51·8% O_2. Rates of oxygen utilization were determined at 40°C, the body temperature of sheep, and were determined while media oxygen content remained between 0·2 and 0·38 ng-atoms/ml. Sterile procedures were used throughout.

L (+)-Lactic acid release and glucose utilization by follicles in culture was determined by enzymic analysis of the media using a Rapid Lactate Stat Pack Kit (Calbiochem., La Jolla, California, U.S.A.), and a glucose analyser (Beckman Instruments, Fullerton, California) respectively.

Measurement of ATP in tissues was achieved by the use of a bioluminescent reaction involving the ATP-dependant luciferin–luciferase system of the firefly, *Photinus phyralis*, as described by Stanley and Williams (1969). ADP and AMP were determined according to the method of Parkinson and Medley (1972).

Immunoreactive oestrogen and progesterone in the culture media were determined as described previously (Seamark *et al.* 1974). Other steroids were analysed by mass spectroscopy as 3-enol-trifluoroacetates or *t*-butyldimethylsilyl ethers, following initial isolation by dextran gel chromatography (R. F. Seamark, G. Phillipou and J. E. A. McIntosh, unpublished data). Results have been expressed on a tissue weight basis. These were determined from mean follicular diameters as described by Weiss *et al.* (1976).

Results

The mean oxygen consumption of the isolated follicles determined after varying periods in organ culture, with or without the addition of hCG, is shown in Table 1. Separation of theca and granulosa tissues revealed that the major part of the oxygen uptake was due to thecal cell activity. Oxygen consumption was little affected by culture for 24 h, but declined significantly ($P < 0·05$) following a further 24 h, then proceeded to rise during the next 48 h. Determination of the oxygen uptake of theca and granulosa components after 72 h in culture revealed a slight, but non-significant, decrease in oxygen uptake by the theca and an increased ($P < 0·05$) uptake by the granulosa. The addition of hCG caused a slight rise ($P < 0·05$) in oxygen consumption in the first 24 h, then tended to have a depressing effect.

Glucose utilization and lactate release by the follicles, as determined by analysis of the culture media, is shown in Table 2. The addition of hCG caused a significant and sustained increase in both oxygen consumption and lactate production by follicles.

The adenine nucleotide content of follicles after varying periods in organ culture is shown in Table 3. No significant change in nucleotide content was seen with time in culture. The effect of hCG on the ATP content of entire follicles and theca and

granulosa tissues separately are shown in Table 4. No differences attributable to hCG treatment were discerned.

Table 1. Effect of hCG on oxygen uptake of sheep ovarian follicular tissues in organ culture

Values shown are means ± s.e.m., and the number of follicles in each group is shown in parentheses. Oxygen uptake is measured per milligram wet weight of tissue per hour. Where indicated, hCG (20 i.u./ml) was added to the culture medium after an initial period of 24 h in culture

Period of culture (h)	Tissue component	Oxygen uptake (umol mg^{-1} h^{-1})		P^A
		No hCG	hCG added	
0	Entire follicle	0·56±0·06 (9)		
	Theca	1·08±0·16 (6)		
	Granulosa	0·05±0·01 (6)		
24	Entire follicle	0·53±0·04 (12)		
48	Entire follicle	0·32±0·02 (9)	0·41±0·02 (7)	0·025–0·05
72	Entire follicle	0·42±0·03 (13)	0·37±0·03 (10)	n.s.
	Theca	0·86±0·14 (6)	1·30±0·22 (6)	n.s.
	Granulosa	0·11±0·02 (6)	0·07±0·02 (6)	n.s.
120	Entire follicle	0·53±0·02 (3)	0·34±0·02 (3)	< 0·01

[A] Calculated for effect of treatment with hCG using Student's *t* test.

Table 2. Effect of hCG on glucose utilization and lactate release by entire sheep ovarian follicles in organ culture

Values shown are means ± s.e.m., and the number of follicles in each group is shown in parentheses. Where indicated, hCG (20 i.u./ml) was included in the culture medium

Period of culture (h)	Glucose utilized (nmol mg^{-1} h^{-1})			Lactate accumulated (μmol mg^{-1} h^{-1})		
	No hCG	hCG added	P^A	No hCG	hCG added	P^A
24	8·5±0·7 (6)	9·6±0·8 (6)	n.s.	16·1±2·0 (6)	14·2±2·9 (6)	
48	5·8±0·4 (6)	9·7±1·1 (6)	<0·01	11·6±2·9 (6)	21·1±1·7 (6)	< 0·01
72	3·2±0·8 (5)	5·9±0·6 (6)	<0·05	9·0±1·7 (5)	13·1±1·0 (6)	< 0·05

[A] Calculated for effect of treatment with hCG using Student's *t* test.

Table 3. Adenine nucleotide content and energy charge ratio (ECR) of sheep ovarian follicles after varying periods in organ culture

Values shown are means ± s.e.m. ECR is measured as (ATP + ½ADP)/(ATP + ADP + AMP)

Period of culture (h)	No. of follicles	Adenine nucleotide content (nmol/mg wet tissue)			ECR
		ATP	ADP	AMP	
0	5	1·4±0·22	0·03±0·02	0·02±0·05	0·97
24	7	2·1±0·16	0·08±0·03	0·18±0·03	0·91
48	4	1·6±0·20	0·01±0·01	< 0·01	0·99

Oestrogen secretion by untreated follicles, as determined by immunoassay, was initially about 100 pmol per milligram tissue per day, and decreased progressively throughout the 5-day period of culture to 50 pmol mg^{-1} day^{-1}; no progesterone

was detected. Treatment with hCG (20 i.u./ml) caused a transitory rise in oestrogen and a dramatic and sustained increase in progesterone production. The results of a more comprehensive analysis of the media of five follicles from each group are shown in Table 5. They reveal that, in addition to oestradiol-17β, the principal oestrogen produced, significant amounts of testosterone, androstenedione and pregnenolone are formed throughout the period of culture by untreated follicles. Treatment with hCG inhibits androgen formation but stimulates the production of pregnenolone and, even more markedly, 20 α-dihydroprogesterone as well as progesterone itself.

Table 4. Effect of hCG on ATP content of sheep ovarian follicles after varying periods in organ culture

Values shown are means ± s.e.m. Where indicated, hCG (20 i.u./ml) was added to the culture medium at time 0

Period of culture (h)	Tissue component	ATP content (nmol/mg)				P^A
		No hCG		hCG added		
0	Entire follicle	1·3±0·2	(10)			
2	Theca	4·6±1·5	(5)	8·1±3·1	(5)	n.s.
	Granulosa	2·8±1·2	(5)	3·4±1·3	(5)	n.s.
24	Entire follicle	2·1±1·6	(7)			
	Theca	1·5	(1)	7·8±2·3	(3)	n.s.
	Granulosa	4·6	(1)	2·8±1·0	(3)	n.s.
48	Entire follicle	1·6±0·2	(4)			
	Theca	5·5±2·7	(4)	2·6±0·6	(3)	n.s.
	Granulosa	2·5±0·3	(4)	2·1±0·1	(3)	n.s.
72	Theca	8·7±0·1	(2)	6·2±2·1	(3)	n.s.
	Granulosa	0·9±0·3	(2)	1·8±0·5	(3)	n.s.

[A] Calculated for effect of treatment with hCG using Student's *t* test.

Discussion

The present study indicates that the isolated sheep ovarian follicle is well maintained in culture under the conditions established by Moor (1973), since all the biochemical parameters determined showed little change throughout the culture period studied.

Little data is available for direct comparison of results, as apart from any species differences most previous biochemical investigations have been made on whole ovaries rather than on individual tissues. Most present information comes from studies on tissues isolated from prepubertal rat ovaries (Ahrén *et al.* 1965, Hamberger *et al.* 1971; Herlitz and Hultborn 1974). Using elegant microtechniques, these workers recorded an oxygen uptake for rat thecal tissues which was about 50% of the value now determined for sheep tissues. However, oxygen uptake of rat granulosa cells (i.e. 2–3 μmol per milligram dry tissue per hour) was 4–6 times greater than that of the sheep, and this activity was further increased by LH.

In earlier studies, Armstrong and Greep (1962) found that the pentose phosphate pathway was not used to any great extent in ovarian tissue, and that glucose was metabolized mainly by glycolysis. This has been confirmed, and it has been subsequently shown that both glucose utilization and lactate acid production were increased by LH in both the entire ovary (Ahrén and Kostyo 1963; Armstrong *et al.* 1963; Hamberger and Ahrén 1967) and the isolated pre-ovulatory follicle (Nilsson 1974).

The finding in the present study that approximately 2 mol lactate accumulates in the culture media containing follicles per mole of glucose utilized indicates that glycolysis is also of major importance to the sheep ovarian follicles.

Table 5. Effect of hCG on steroid production by sheep ovarian follicles in culture

Values shown are means ± s.e.m. of five replicates. Where indicated hCG (20 i.u./ml) was included in the culture medium

Steroid		Steroid released [pmol (mg tissue)$^{-1}$ (24 h)$^{-1}$] on					
		Day 1		Day 2		Day 3	
		No hCG	hCG added	No hCG	hCG added	No hCG	hCG added
C21	Pregnenolone	9 ± 3	94 ± 62	28 ± 12	101 ± 63	9 ± 6	79 ± 50
	Progesterone	0	96 ± 25	0	290 ± 35	0	548 ± 89
	20α-dihydro-progesterone	0	95 ± 25	0	760 ± 70	0	2170 ± 90
C19	Dehydroepiandrosterone	0·3 ± 0·3	6 ± 3	0	0	0	0
	Androstenedione	48 ± 6	41 ± 6	47 ± 10	0	35 ± 3	0
	Testosterone	210 ± 59	45 ± 10*	223 ± 42	0	164 ± 27	0
C18	Estrone	5 ± 3	4 ± 3	5 ± 2	0	9 ± 3	0
	Estradiol-17β	92 ± 30	350 ± 102*	73 ± 25	165 ± 44*	47 ± 14	47 ± 22

* $P < 0{\cdot}05$ (i.e. hCG treatment had a significant effect).

The increase in glucose utilization and lactate accumulation following LH or hCG treatment in both rat and sheep corresponds with the transformation of the follicle from secreting oestrogens and androgens to secreting mainly progestins (T. Hillensjo, S. Bauminger and K. Ahrén, personal communication), as shown by the results in Table 5. We have previously proposed that these changes in steroidogenesis represent the transfer of steroid synthetic capacity from the theca interna to the membrana granulosa (Moor *et al.* 1973), and it is tempting to suggest that the increase in glycolytic activity is attributable to the granulosa cells as they assume their new role. It has been previously suggested from data derived from experiments on the adrenal gland (Haynes and Berthet 1957) that hormonal stimulation of glycolysis might be an early step in the sequence of events leading to increased steroidogenesis.

Whether hCG stimulates lactate production by theca or granulosa cells or both remains to be determined. Hamberger (1968) found that LH stimulated the respiration of isolated granulosa cells but not of thecal cells, whilst FSH had a reverse effect. However, in a recent study on the effects of LH on isolated sheep ovarian follicles, cyclic AMP formation, which increases rapidly in follicles exposed to LH, occurred predominantly in the thecal compartment (Weiss *et al.* 1976) and this result agrees with other evidence that the receptor site for LH in both sheep (R. M. Moor, unpublished data) and rat follicles (Zeleznik *et al.* 1974) is situated in the theca interna.

In studies on the possible means whereby gonadotrophins regulate glycolysis the importance of the adenine nucleotides has been emphasized. Ahrén *et al.* (1965) observed that addition of LH to isolated rat ovaries resulted in decreased tissue ATP

levels, and they postulated that this was likely to be one of the factors which lead to increased activity of a key regulatory enzyme phosphofructokinase.

In the present experiments prolonged exposure of the isolated sheep follicles to hCG was without significant effect on the tissue ATP content. The mean value of tissue ATP content found from the sheep is very similar to that described for bovine follicular tissue by Stahler and Huch (1971). However, it should be noted that the ADP content of the sheep follicle was less than one-tenth of that of the bovine tissue, indicating a higher energy charge ratio (ECR) (Atkinson 1968) for the sheep follicle. This high ECR, and thus presumably the overall magnitude and direction of cellular pathways in the sheep follicle, was sustained throughout the period of culture.

In a previous paper we reported differences in both the pattern and amounts of steroid hormone released in culture by follicles collected at different stages of the cycle (Seamark *et al.* 1974). The pooling of follicles from days 4 to 14 for the present study has undoubtedly contributed to the wide variation in values and responses noted between the different follicles. Studies with follicles obtained from ewes at known mating dates are presently in progress to ascertain whether this variation has masked the detection of more subtle changes and responses in the biochemical parameters monitored.

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