Influence of follicular maturation on 3-hydroxy-methylglutaryl coenzyme A reductase activity in hen granulosa cells*

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Summary. The activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase: EC 1.1.1.34) was measured in a microsomal preparation of the granulosa of rapidly growing ovarian follicles of laying hens in the late preovulatory period (2–3 h before expected ovulation). The specific activity of the enzyme was measured in the five largest (F_1 – F_5) preovulatory follicles, F_1 being the follicle destined to ovulate first. Enzyme activity increased concomitantly with follicle size. The apparent K_m of the enzyme decreased 60–80% from the smallest to the largest preovulatory follicle. There was no significant change in the V_{max} during follicle development. Although our results have demonstrated the presence of HMG/CoA reductase in chicken granulosa cells and the progressive increase of its activity with follicular maturation, the quantitative significance of de-novo synthesized cholesterol as steroid hormone precursor remains to be ascertained.

Introduction

The importance of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase: EC 1.1.1.34), the rate-limiting enzyme in cholesterol biosynthesis (Beg & Brewer, 1981), has been examined in relation to ovarian steroidogenesis in several mammalian species (reviewed by Gwynne & Strauss, 1982). Cholesterol, the common precursor of all steroid hormones, is made available in steroidogenic cells from (a) de-novo synthesis, (b) lipoprotein-carried cholesterol from the circulation, and (c) intracellular stored cholesterol.

Several studies performed under in-vivo conditions have demonstrated that the steroidogenic cholesterol substrate of adrenals, corpus luteum, placenta and ovary of humans (Borkowski *et al.*, 1967; Hellig *et al.*, 1970), rats (Major *et al.*, 1967) and dogs (Krum *et al.*, 1964) originates from extracellular sources. However, rat and hamster Leydig cells, hamster adrenal gland and Leydig tumour cells preferentially utilize endogenously synthesized cholesterol as the primary substrate for steroidogenesis (Morris & Chaikoff, 1959; Lehoux & Lefebvre, 1980). Silavin & Strauss (1983) have reported that hamster granulosa cells depend solely on prestored endogenous cholesterol for acutely stimulated steroidogenesis, emphasizing the fact that different steroidogenic tissues from different species may depend on different sources of cholesterol for steroid hormone biosynthesis. Avian granulosa cells, particularly those isolated from the mature follicle of laying hens, produce copious amounts of progesterone in response to LH and other agonists during short-term incubations (Asem & Hertelendy, 1985). However, the steroidogenic response of granulosa cells harvested from younger, developing follicles to LH or to nonreceptor-mediated agonists such as forskolin and cyclic AMP analogues is greatly reduced (Asem & Hertelendy, 1985). It therefore

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appears that the steps responsible for the differential steroidogenic ability of mature and developing avian granulosa cells obtained in the late preovulatory period are distal to cyclic AMP generation, with the cholesterol side-chain cleavage enzyme step as one of the main control points. Other regulatory steps may impinge on cholesterol synthesis and/or storage, release and transportation to the mitochondrial side-chain cleavage enzyme site.

In the present studies, we measured the activity of HMG-CoA reductase, the key enzyme in cholesterol biosynthesis, in the granulosa cell layer of the chicken and correlated it with follicle size.

Materials and Methods

Hormones and chemicals. DL-3-Hydroxy-3-methylglutaryl CoA (HMG-CoA), DL-mevalonic acid lactone, glucose-6 phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP⁺), glucose-6 phosphate and sucrose were purchased from Sigma Chemical Co. (St Louis, MO). Silica gel GHL thin-layer plates were obtained from Analtech (Newark, DE). Radiolabelled compounds DL-3-[glutaryl-3-¹⁴C]hydroxy-3-methylglutaryl coenzyme A (sp. act. 47.2 mCi/mmol), RS-[5-³H(N)]mevalonolactone (sp. act. 24.0 Ci/mmol), and [1,2,6,7-³H]progesterone (sp. act. 90.1 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Compactin (ML-263B) was obtained through the courtesy of Dr A. Endo, Tokyo Noko University, Tokyo, Japan, and of Dr J. F. Strauss III, University of Pennsylvania, Philadelphia, PA.

Experimental animals. White Leghorn hens in their first year of egg laying were caged individually, in a windowless air-conditioned room with lights on from 06:00 to 20:00 h. The birds had free access to a pelleted commercial laying ration (Purina Layena) and tap water. The time of oviposition was monitored by an electronic device (Hammond *et al.*, 1980) and birds with at least 6 consecutive laying days were selected for experiments.

All animals used in this study were killed 2–3 h before ovulation of the largest follicle. During this period of the ovulatory cycle, granulosa cells are very sensitive to hormonal stimulation (Hertelendy & Asem, 1984).

Preparation of tissue. Granulosa tissue obtained as described by Asem & Hertelendy (1985) was homogenized in 5 volumes of 20 mM-potassium phosphate buffer, pH 7.4, containing 200 mM-potassium chloride, 5 mM-EDTA, 0.2 M-sucrose and 5 mM-dithiothreitol (homogenizing buffer), in an all-glass dounce homogenizer. The homogenate was centrifuged at 600 g for 60 min and the resulting supernatant fraction was then centrifuged at 100 000 g for 60 min to sediment microsomes. The microsomes were washed once in homogenizing buffer before resuspension in assay buffer.

Enzyme assay. The HMG-CoA reductase activity was measured by the radiochemical assay described by Balasubramanian *et al.* (1977), with slight modifications. Briefly, the microsomes were resuspended in assay buffer containing 100 mM-potassium phosphate, pH 7.4, 5 mM-EDTA and 5 mM-dithiothreitol. The final mixture contained 2.5 mM-NADP⁺, 20 mM-glucose-6 phosphate, 1 unit of glucose-6 phosphate dehydrogenase, and appropriate aliquants of microsomal protein. Final incubation volume was 200 µl. Tubes were preincubated for 10 min at 37°C, after which the reaction was initiated by the addition of DL-3-[glutaryl-3-¹⁴C]hydroxy-3-methylglutaryl coenzyme A (20 000 d.p.m./nmol). The reaction was allowed to continue for an additional 8 min at 37°C, after which it was terminated with 20 µl 5 N-HCl containing [5-³H]mevalonic acid (10⁵ d.p.m./µmol) to monitor recovery of the product. The tubes were incubated for an additional 30 min at 37°C to ensure lactonization of [¹⁴C]mevalonic acid. Blank tubes were treated identically except that stopping solution was added before incubation.

The resulting [¹⁴C]mevalonolactone product was extracted twice with 40 volumes of diethyl ether. The ether phase was dried under nitrogen and the product was separated by thin-layer chromatography in a solvent system of toluene:acetone:acetic acid (20:10:1; by vol.). The silica gel was scraped from the plates in 1-cm strips and the radioactivity in every strip was measured in a Beckman liquid scintillation spectrophotometer (Model LS 7000) in a toluene:triton (2:1; v/v) scintillation cocktail. The radioactivity corresponding to an authentic mevalonolactone spot (*R_f* 0.6–0.8) was used in calculating the amount of mevalonic acid formed.

Recovery of product averaged 65–75% and values reported have been corrected accordingly. The enzyme assay conditions were selected such that the product formation was linear with time and protein concentration. Results are expressed as nmol mevalonate/mg protein/min.

Protein content was measured by the method of Bradford (1976) using BSA as standard.

Data analysis. Data were tested for statistical significance by analysis of variance and post-hoc Tukey's test. Student's *t* test was used when applicable.

Results

The activity of HMG-CoA reductase increased concomitantly with follicular maturation (Fig. 1). Its activity increased 4–6-fold from the F₅ to the F₁ follicle. Using increasing substrate concentrations, typical hyperbolic saturation curves were obtained (Fig. 2). Double-reciprocal plots of the

Fig. 1. Effect of follicle size on HMG-CoA reductase activity. Microsomes isolated from granulosa membranes obtained from the five largest preovulatory follicles (F₁–F₅) 2–3 h before expected ovulation were incubated with 10 μ M-HMG-CoA. Protein concentration was 7–9 μ g/tube. All other conditions were as described in 'Materials and Methods'. Results are mean $\pm$ s.e.m. of 4 separate experiments each performed in triplicate. * $P < 0.05$ compared with value for F₁ follicle.

data indicated that there was a significant change in the apparent K_m of the enzyme as the follicle approached ovulation (Fig. 3). To obtain the apparent kinetic parameters (K_m and V_{max}) the data were subjected to analysis by Hofstee's method (1952) and a linear regression programme was used to calculate the slope (the negative value of apparent K_m) and the y-intercept (the apparent V_{max}). Table 1 shows that the apparent K_m decreased 80% during follicular development from the F₄ to the F₁ follicle (65 μ M and 10 μ M), whereas the corresponding V_{max} values increased only 1.5-fold.

The amount of mevalonate (product) formed by microsomes in our incubations was directly proportional to the quantity of the microsomal protein up to 10 μ g. For F₁ cells, mevalonate formed by 1, 3, 6 and 10 μ g microsomal protein was 14.6 ± 1.4 , 27.0 ± 6.1 , 36.6 ± 2.6 and 42.0 ± 1.0 nmol/min respectively. When microsomes were isolated from F₃ cells, product formation by similar amounts of protein was 8.75 ± 0.2 , 11.5 ± 0.9 , 21.9 ± 1.0 and 26.5 ± 2.0 nmol/min respectively. The product formation progressed at an approximately linear rate up to 12 min for F₁ and 20 min for F₃ follicles (Fig. 4), manifesting once again the distinct difference in enzyme activity between mature and developing follicles.

Compactin, a specific inhibitor of HMG-CoA reductase (Endo *et al.*, 1977), inhibited the enzyme activity such that 12.5 μ M and 25 μ M compactin suppressed the enzyme activity from 5.9 ± 0.8 nmol/mg protein/min to 0.4 ± 0.001 and 0.32 ± 0.02 nmol/mg protein/min respectively ($n = 6$ observations).

Fig. 2. Influence of follicular maturation of HMG-CoA reductase activity. Microsomes, isolated from granulosa membranes obtained from the 4 largest preovulatory follicles (F_1 – F_4) 2–3 h before expected ovulation, were assayed for enzyme activity in the presence of increasing concentrations of HMG-CoA substrate. Protein concentration was 7 μ g/reaction tube. Results are mean $\pm$ s.e.m. of triplicate observations. The vertical bars representing s.e.m. are not shown because these were smaller than the size of the symbols. The experiment was repeated once more with similar results. The F_3 and F_4 curves are significantly different from the F_1 curve ($P < 0.05$).

Fig. 3. Lineweaver-Burk plots of the HMG-CoA reductase activity. The values shown in Fig. 2 are presented in a double reciprocal plot. The straight lines were calculated using linear regression analysis. All four lines had correlation coefficients > 0.9 .

Table 1. Effect of follicular maturation on the kinetic parameters of HMG-CoA reductase activity

Follicle	K_m (μM)	$V_{\max}$ ($\text{nmol} \cdot \text{min}^{-1}$ $\cdot \text{mg protein}^{-1}$)	Correlation coefficient	Regression equation
F ₁	15.0	13.2	0.95	$y = 13.2 - 15.0x$
F ₂	20.5	14.8	0.94	$y = 14.8 - 20.5x$
F ₃	47.0	11.8	0.91	$y = 11.8 - 47.0x$
F ₄	65.0	8.0	0.90	$y = 8.0 - 65.0x$

Enzyme assay was performed on microsomal fraction as described in 'Materials and Methods'.

The K_m and $V_{\max}$ values were estimated using Hofstee plots from data shown in Fig. 2.

Fig. 4. Time course of HMG-CoA reductase activity. Microsomes obtained from granulosa membranes of follicles removed 2–3 h before ovulation were incubated with $10 \mu\text{M}$ -HMG-CoA. At the times indicated the reaction was stopped in some samples and treated as described in 'Materials and Methods'. Values presented are the mean $\pm$ s.e.m. of triplicate incubations. The experiment was repeated and gave similar results.

Discussion

One of the significant findings of this study is the demonstration that HMG-CoA reductase activity increased with follicular development. Although there was a small increase in the apparent $V_{\max}$ with follicle size, the high specific activity of the enzyme in F₁ and F₂ follicles was due primarily to a 60–80% decrease in the apparent K_m as the follicles underwent maturation during the last few days of their lifespan. Ovarian HMG-CoA reductase activity has been shown to increase during pregnancy in rabbits (Kovanen *et al.*, 1978) as well as in the ovary of the pseudopregnant rat (Schuler *et al.*, 1979). The increase in enzyme activity under these conditions has been interpreted to reflect physiological responses of the ovary to increased demand of cholesterol for steroidogenesis.

Progesterone, which plays a key role in the daily ovulatory cycle of the hen, is derived principally from the largest (F_1) follicle (Etches *et al.*, 1981). The ability of F_1 granulosa cells to respond to the preovulatory LH rise has been generally attributed (albeit by implication) to an increase in LH receptors and to the activity of LH receptor-coupled adenylate cyclase (Calvo *et al.*, 1981). However, Asem & Hertelendy (1985) have indicated that the activity of cholesterol side-chain cleavage enzyme (a critical step in the biosynthesis of progesterone from cholesterol), as well as that of the high K_m β -hydroxysteroid dehydrogenase, increases significantly during follicular maturation. Therefore, the ability of mature granulosa cells to produce progesterone can be attributed at least in part to such increments in the quantity of steroidogenic enzymes during the last few days of follicular development. In contrast, the present studies suggest that HMG-CoA reductase undergoes qualitative (decrease of K_m) rather than quantitative changes during follicular maturation.

The fact that the granulosa cells of a single F_1 follicle are able to produce microgram quantities of progesterone per hour during short-term culture in response to appropriate stimulation, even in the absence of exogenous lipoprotein cholesterol (Asem & Hertelendy, 1985) has suggested that large intracellular stores of cholesterol are present or active de-novo sterol synthesis takes place during such stimulation in combination with endogenous cholesterol mobilization.

The results obtained in the present studies do not permit distinction of these two possible events. However, Wells *et al.* (1981) have reported that, in fowl granulosa cells isolated in the late preovulatory period, progesterone production from endogenous precursors was over 60 times greater than that synthesized *de novo* from ^{14}C -labelled substrates (acetate, octanoate, mevalonic acid lactone and glucose). Wells *et al.* (1981) concluded that cholesterol substrate utilized for progesterone synthesis in chicken granulosa cells *in vitro* did not originate from a newly synthesized pool. Preliminary studies (unpublished) in our laboratory seem to support the conclusion of Wells *et al.* (1981), because compactin in the dose range that inhibited HMG-CoA reductase activity had no significant effect on basal or LH-stimulated progesterone synthesis *in vitro*.

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