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THE DIFFERENTIAL DEVELOPMENT OF MITOCHONDRIAL CYTOCHROME *P*-450 AND THE RESPIRATORY CYTOCHROMES IN RAT OVARY

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Summary

The cytochrome concentrations in ovarian mitochondria were determined in immature and superovulated rats. The respiratory cytochromes (b, $c + c_1$ and $a + a_3$) were found to increase approximately 3-fold during the transition from immature follicle to active steroid-producing corpus luteum. At the same time, mitochondrial cytochrome *P*-450 exhibited a 10-fold increase in concentration, reflecting the ability of the gland to synthesize steroids. This different rate of synthesis of mitochondrial cytochrome *P*-450 and respiratory cytochromes may be regarded as a biochemical differentiation step necessary for the granulosa cells to develop steroidogenic competence.

Introduction

The corpus luteum of the ovary, like other steroid-producing tissues, is known to contain two electron transport chains in its mitochondria: the ubiquitous energy-producing chain terminating with cytochrome oxidase, and the chain utilising cytochrome *P*-450 as the terminal oxidase which catalyses the conversion of cholesterol to the steroid, pregnenolone [1]. This latter reaction is the rate-limiting step in steroidogenesis. The two chains are interrelated in that the cytochrome-*P*-450-dependent cholesterol side-chain-cleavage reaction can be controlled by varying the rate of flow of electrons through the respiratory cytochromes [2]. Compared with the corpus luteum, the immature ovarian follicle has a very low rate of cholesterol conversion [3] and, accordingly, its rate of steroid synthesis is low. The transition from the relatively inactive follicle to the very active corpus luteum is accompanied by a large increase in tissue mass. This growth and development of the follicle into the corpus luteum,

together with the increased biosynthesis of steroids, places an increased demand on the electron transport chains to be available both for energy and steroid production. Our aim was to investigate the relationship between these two electron transport chains, particularly the concentrations and activities of the terminal oxidases, during this development from follicle to corpus luteum. The model used was the immature rat ovary treated with gonadotropins [3].

Methods and materials

Preparation of Mitochondria. Female Wistar rats (20-days-old) were injected subcutaneously with 50 I.U. pregnant mare's gonadotropin. 48 h later they were injected subcutaneously with 25 I.U. human choriogonadotropin. A sample number of rats was killed each day and their ovaries dissected out. The ovaries were washed in iced 0.25 M sucrose, cleaned of adipose tissue and oviducts, weighed, and then homogenised in a glass and Teflon homogeniser. Cells, nuclei and debris were removed by centrifuging at 850 × *g* for 10 min. The supernatant was recentrifuged at 1000 × *g* for 10 min to remove any debris or cells unavoidably transferred in the initial step. The mitochondrial fraction was collected by centrifugation at 8500 × *g* for 10 min, washed in 1.54 M KCl and resuspended in a buffer, pH 7.4, containing 20 mM KCl, 10 mM potassium phosphate, 15 mM triethanolamine hydrochloride, 5 mM $MgCl_2$ and 0.25 M sucrose. This buffer was used throughout.

Preparation of Microsomal Fraction. The 8500 × *g* supernatant obtained from the mitochondrial preparation was centrifuged at 15 000 × *g* for 15 min and the pellet discarded. The microsomal fraction was collected from the supernatant in the Beckman L5-65 ultracentrifuge at 105 000 × *g* for 30 min. The pellet was washed in the above buffer and again collected at 105 000 × *g* for 30 min, and resuspended in the assay buffer.

Measurement of Cytochrome Concentrations. The concentrations of cytochrome oxidase, cytochrome $c + c_1$ and cytochrome *b* were measured using their reduced minus oxidized spectra as described by Chance and Williams [4]. Calculations of the concentrations were based on the millimolar extinction coefficient of $\epsilon^{mm}_{605-630} = 19.4$ [5] for cytochrome oxidase $\epsilon^{mm}_{562-575} = 20$ for cytochrome *b* and $\epsilon^{mm}_{551-540} = 19$ for cytochrome $c + c_1$ [6]. It was recognised that the measurement of cytochromes *b* and *c* were influenced slightly by the presence of cytochrome *P*-450 which is a *b* cytochrome [1]. The reducing agent was sodium dithionite. Reduced minus (reduced + CO-treated) mitochondria were used to assay cytochrome *P*-450 concentrations, the millimolar extinction coefficient employed being $\epsilon^{mm}_{450-490} = 91$ [7]. Any haemoglobin contamination was detected from the CO difference spectrum of aerobic mitochondria. Only mitochondria without haemoglobin contamination were used for the spectral studies. Mitochondrial preparations were maintained at a protein concentration of about 0.6 mg/ml. The results were only reproducible if the turbidity of the mitochondrial suspensions was kept approximately the same. An increase in turbidity resulted in a calculable overestimate of cytochrome concentration (Naumoff, P.A. and Stevenson, P.M., unpublished results).

Microsomal cytochrome *P*-450 was also assayed by the reduced CO difference spectrum as for mitochondrial cytochrome *P*-450.

Measurement of Cytochrome Oxidase Activity. The activity of cytochrome oxidase was assayed by the method of Yonetani [8] in which the rate of oxidation of reduced cytochrome *c* was a function of cytochrome oxidase activity. The reaction mixture contained 300 μl of a 1% solution of reduced cytochrome *c* in a total volume of 3.0 ml 0.1 M phosphate buffer, pH 6.0, at 37°C. The reaction was followed spectrophotometrically at 550 nm after the addition of 50 μl (approx. 30 μg) of the mitochondrial suspension. The rate was calculated using the millimolar extinction coefficient for cytochrome *c* of 29.4 at 550 nm [9].

The Activity of Cytochrome P-450. An indication of the activity of the cytochrome-*P*-450-containing electron transport chain was gained by measuring the rate of [4-^{14}C]cholesterol conversion to [4-^{14}C]pregnenolone and [4-^{14}C]progesterone by isolated mitochondria. These assays were performed by S.P. Klinken in our laboratories [3].

Mitochondrial protein. This was estimated by the Ponceau S method of Pesce and Strande [10].

Materials. Cytochrome *c*, purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), was reduced by reaction with ascorbic acid in 0.1 M potassium phosphate buffer, pH 7.0. Excess ascorbate was removed by dialysis in 0.1 M potassium phosphate buffer, pH 7.0. Pregnant mare's gonadotropin (folligon) was from Intervet (Artarmon, New South Wales, Australia) and human chorionic gonadotropin was obtained from Parke Davis and Co. (Sydney, New South Wales, Australia). All other reagents were of analytical grade.

Results

Treatment with pregnant mare's gonadotropin initiated a sequence of events leading to increases in ovarian size, cytochrome concentrations, and steroid production. Mitochondrial cytochrome *P*-450 increased from 1.24 to 169 nmol per ovary during the 6 days after treatment with pregnant mare's gonadotropin, representing a 136-fold increase. This is illustrated in Fig. 1 which also shows the development of cholesterol side-chain-cleavage activity, which is dependent on mitochondrial cytochrome *P*-450 [3]. Simultaneously, total cytochrome oxidase increased 25-fold from 4.65 nmol in the untreated ovary to 118 nmol by the 6th day after gonadotropin (Fig. 1).

While the absolute amounts of cytochrome in nmol per ovary showed the extent of their huge de novo synthesis, there was also a concomitant increase in mitochondrial protein per ovary. Therefore, the increase in cytochromes, when expressed as specific activity, was less dramatic. Table I shows that cytochrome *P*-450 rose from 0.03 μmol/g to 0.29 μmol/g mitochondrial protein, while cytochrome oxidase rose from 0.08 μmol/g mitochondrial protein to 0.24 μmol/g mitochondrial protein during the 6-day period. This represents a 10-fold increase in mitochondrial cytochrome *P*-450 concentration and a 3-fold increase in cytochrome oxidase concentration. The daily increases in cytochrome *P*-450 were significant ($P = 0.01$) for the first 4 days after gonadotropin treatment, but not for the changes between days 4 and 5, and between days 5 and 6. There was no significant daily increase in cytochrome oxidase concentration, although the total increase from untreated to 6 days after treatment with

TABLE I

THE CONCENTRATIONS OF CYTOCHROMES AND THE ACTIVITY OF CYTOCHROME OXIDASE IN IMMATURE RAT OVARIES, INDUCED WITH GONADOTROPINS

Concentrations are μmol/g mitochondrial protein, and activity is μmol ferrocytochrome *c* oxidized/g mitochondrial protein per min at 37°C.

	Concentration (mean ± S.D., n = 5)					Activity (mean ± S.D., n = 3)
Days after pregnants mare's gonadotropin	Cytochrome *P*-450	Cytochrome oxidase ($a + a_3$)	Cytochrome ($c + c_1$)	Cytochrome *b*	Cytochrome, *P*-450 cytochrome oxidase ratio	Cytochrome oxidase
0	0.03 ± 0.02	0.08 ± 0.02	0.14 ± 0.01	0.10 ± 0.10	0.4	2.83 ± 0.8
1	0.07 ± 0.03	0.15 ± 0.06	0.15 ± 0.01	0.10 ± 0.12	0.5	3.51 ± 0.5
2	0.17 ± 0.03	0.19 ± 0.10	0.17 ± 0.01	0.12 ± 0.09	0.9	4.43 ± 0.2
3	0.20 ± 0.04	0.19 ± 0.07	0.19 ± 0.03	0.17 ± 0.08	1.0	4.76 ± 0.4
4	0.24 ± 0.03	0.21 ± 0.09	0.23 ± 0.10	0.23 ± 0.11	1.1	5.52 ± 0.1
5	0.29 ± 0.04	0.24 ± 0.05	0.29 ± 0.05	0.26 ± 0.10	1.2	7.34 ± 0.1
6	0.28 ± 0.04	0.22 ± 0.04	0.31 ± 0.04	0.29 ± 0.03	1.3	7.70 ± 1.5

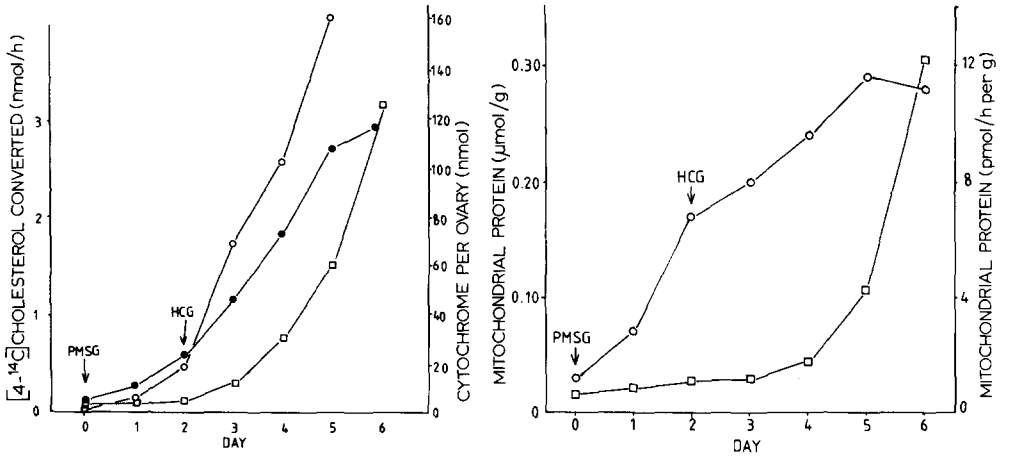

Fig. 1. Total mitochondrial cytochrome *P*-450 (○) and cytochrome oxidase (●), and the rate of steroidogenesis (□) per ovary during development. The cytochromes were measured by difference spectroscopy as described in the text. Steroidogenesis was the rate of formation of [4-^{14}C]pregnenolone and [4-^{14}C]progesterone from [4-^{14}C]cholesterol by isolated mitochondria [2]. Values shown are the mean of five experiments. PMSG, pregnants mare's gonadotropin; HCG, human choriogonadotropin.

Fig. 2. Concentration of mitochondrial cytochrome *P*-450, and the specific activity of the cholesterol side-chain-cleavage enzyme. Cytochrome *P*-450, ○, (left axis), was measured by the reduced —CO difference spectrum as described in the text, while cholesterol side-chain-cleavage, □, (right axis) was calculated from data supplied by S.P. Klinken where [4-^{14}C]cholesterol was incubated with isolated mitochondria in the presence of isocitrate as the energy source [2,3]. PMSG, pregnant mare's gonadotropin; HG, human choriogonadotropin.

pregnant mare's gonadotropin was significant ($P = 0.01$).

The values obtained on days 2 and 3 after initial gonadotropin injection were similar to those obtained by McIntosh et al. [11]; namely 0.17 and 0.23 nmol/mg mitochondrial protein for cytochromes *P*-450 and oxidase, respectively. Note, these authors use the millimolar extinction coefficient $\epsilon^{mm}_{605-625} = 16$ for cytochrome oxidase whereas we used $\epsilon^{mm}_{605-630} = 19.4$. Our results for cytochrome concentrations in the corpus luteum also compared well with those obtained by Cooper and Thomas [1] after treatment with pregnant mare's gonadotropin and human choriogonadotropin.

Contamination of the mitochondrial fraction with cytochrome *P*-450 derived from endoplasmic reticulum was minimal because the concentration of cytochrome *P*-450 present in the microsomal fraction was very low (less than 0.03 μmol/g protein). This concentration varied very little throughout the entire 6 day sequence and, therefore, the amount of extra-mitochondrial cytochrome *P*-450 contributing to our results was negligible. Indeed, contamination of the microsomal fraction by mitochondria, as measured by cytochrome oxidase activity, accounted for up to 0.02 μmol cytochrome *P*-450/g microsomal protein.

Table I shows that the increase in cytochrome oxidase activity, estimated from the rate of oxidation of ferrocytochrome *c*, was similar to the increase in its concentration. The activity increased 2.7-fold during the 6 day period from 2.83 μmol/min per g protein to 7.70 μmol cytochrome *c* oxidised/min per g

mitochondrial protein. The specific activity of cholesterol side-chain-cleavage, which is dependent on the activity of cytochrome *P*-450, increased 22-fold during the same time period (calculated from data supplied by S.P. Klinken, see Fig. 2). However, this enzyme is controlled by the rate of supply of substrate by choriogonadotropin [12] and, therefore, a better index of the activity of the cytochrome *P*-450 complex would be obtained when the rate of cholesterol side-chain-cleavage is maximally stimulated by the acute injection of choriogonadotropin: this increase was about 35-fold (Stevenson, P.M. and Klinken, S.P., unpublished results).

During the 6 day period when the terminal oxidases were increasing in concentration and activity, there was also an increase in concentration of cytochrome *b* of 2.9-fold, from 0.10 to 2.9 μmol/g mitochondrial protein, and of cytochrome $c + c_1$ of 2.2-fold, from 0.14 μmol/g to 0.31 μmol/g mitochondrial protein (see Table I). The lower increases in cytochrome $c + c_1$ may result from loss of soluble cytochrome *c* during the subfractionation procedure, whereas the cytochrome *b* might be overestimated slightly due to the presence of cytochrome *P*-450. It was considered that the exact measurement of these cytochromes was not important to the study. There was no significant daily increase in either of these two cytochromes but there was a significant overall increase ($P = 0.01$) during the 6 day period.

Discussion

The immature ovary contains very little cytochrome *P*-450, which is reflected by its inability to produce steroids. The results show that treatment with gonadotropin initiated synthesis of mitochondrial cytochrome *P*-450 and cytochrome oxidase as well as other mitochondrial proteins. Most important, there was a differential rate of synthesis of the cytochromes in the two electron transport chains: there was a 10-fold increase in concentration of cytochrome *P*-450 and in the same mitochondria only 3-fold increase in the cytochromes of the respiratory chain, i.e. cytochromes *b*, $(c + c_1)$, $(a + a_3)$. The 3-fold increase probably represents the synthesis of constitutive enzymes necessary to supply the energy for growth of the tissue. However, the 10-fold increase in cytochrome *P*-450 may represent a biochemical differentiation step in the mitochondria; a synthesis of enzymes necessary for the specific function of the tissue, i.e. to produce steroids. There are two cell types in the immature follicle, thecal and granulosa. Initially only thecal cells are capable of cholesterol utilization [13] and the small amount of cytochrome *P*-450 measured in untreated ovaries is probably of thecal origin. The acquisition of cytochrome *P*-450 by the granulosa cells, therefore, represents the differentiation of cells from non-progesterone-producing ones to those which can synthesize progesterone. Klinken and Stevenson [3] found a similar development or differentiation of cholesterol side-chain-cleavage in the same rat model. Our work has shown that development of cytochrome *P*-450 is much earlier than cholesterol conversion to steroids. Therefore, mitochondria have part, at least, of the apparatus required for cholesterol utilization before the sharp increase in steroid production commences by the corpus luteum (Figs. 1, 2). A transport system allowing cholesterol to reach mitochondrial cytochrome *P*-450 is also necessary

for cholesterol side-chain-cleavage. This may develop more slowly than the cytochrome *P*-450, accounting for the lag between the development of cytochrome *P*-450 concentration and cholesterol side-chain-cleavage activity (Fig. 2). More relevant to this paper, Robinson and Stevenson [2] showed that there must be a balanced flow of electrons between the respiratory and cytochrome *P*-450 chains before steroidogenesis can occur. It appears that this balance is attained when cytochrome oxidase and cytochrome *P*-450 are present in equimolar amounts, i.e., on day 3, immediately before ovulation, when the rate of steroid synthesis begins to accelerate (Table I, Fig. 2).

The literature [14] suggests that the rate of steroidogenesis during development is limited by the numbers of lutropin or choriogondadotropin receptors on the cell surfaces. However, steroidogenesis cannot occur if the enzyme complement of the cell is incomplete. We have shown that cytochrome *P*-450 is present in only very small amounts in the undeveloped follicle and that the de novo synthesis of mitochondrial cytochrome *P*-450 is initiated, directly or indirectly, by pregnant mare's gondadotropin. This de novo synthesis of mitochondrial enzymes is just as necessary for the cell to gain steroidogenic competence as the development of lutropin receptors. They are both part of the very complex apparatus essential for steroid hormone production and their development may be initiated by the same mechanism.

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