VITELLOGENIN SYNTHESIS IN ISOLATED HEPATOCYTES

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1. Introduction

It is well known that in birds and in amphibians the production of egg yolk proteins in liver is controlled by estrogen. In vivo the synthesis of vitellogenin can be artificially induced by a single injection of estradiol into male or immature animals [1–4]. The molecular events which precede the appearance of vitellogenin in the circulation are partially known [5–7] but the mechanism is far from being completely understood.

The development of an in vitro system to study in detail such molecular reactions is clearly a justifiable and challenging need. Relative to the in vivo situation where we are dealing with a lag period of more than 3 h [7,8] before phosvitin or lipovitellin synthesis can be measured, such in vitro incubations present among others the technical problem of long-term survival of the cells. Primary organ cultures have been quite successful in this regard. Green and Tata [9] using explants of amphibian liver have developed a system which shows the in vivo response to estradiol also in vitro. Such a system has not yet been described with avian liver, although attempts are being made in this direction. The group of Carinci reported to have induced the synthesis of phosvitin by means of adding estrogen to primary cultures of embryonic chick liver [10]. Isolated chick hepatocytes are reported here for the first time in a study of their ability to synthesize vitellogenin in vitro. We show that the isolated cells metabolized added 17β-estradiol and doubled the amount of nuclear estrogen receptor within 10 min, but only if the hormone was present in the cells before their isolation did they continue in vitro to produce more protein than the controls. By immunoprecipitation synthesis of vitellogenin was demonstrated. The findings suggest that to promote the sequence of biochemical events leading to vitellogenin synthesis an unknown factor may be essential at a time of cell to cell contact.

2. Materials and methods

2.1. *Isolation of hepatocytes*

Male chicks weighing 300–500 g were used. The liver was perfused with collagenase according to Krebs et al. [11]. Hepatocytes were washed and suspended in buffered Krebs-Henseleit saline, pH 7.4, at a concentration of approximately 5 mg protein/ml. When the effect of estradiol was to be tested in the perfusate, it was added as 1 mg dissolved in 0.1 ml of 2,3-butane diol at the beginning of the perfusion.

2.2. *Incubation of hepatocytes*

Isolated hepatocytes (1 ml) were incubated in a mixture containing 0.5 ml of 10% albumin in buffered Krebs-Henseleit saline, 0.02 ml of 1% aqueous streptomycin, 0.025 ml of 10% aqueous penicillin and saline to 2 ml. For testing the in vitro effect of estradiol, 10^{-6} M 17β-estradiol in 0.2% DMSO or 2×10^6 cpm [^{3}H]estradiol in 0.2% DMSO were also present. For the investigation of protein synthesis 0.2 ml of an amino acid mixture [12] and 0.5 μCi [^{14}C]leucine (10 mCi/mmol) were included in the above mixture. Hepatocytes were always added last to the incubation mixture contained in 25-ml Erlenmeyer flasks. These were gassed with 5% CO_2–95% O_2, stoppered and placed in a shaking water bath at 37°C. After the incubation the contents of the flasks were quickly

transferred into specially designed separation tubes containing 0.4 ml of 20% perchloric acid [13]. A rapid centrifugation of less than 1 min in a table top centrifuge separated cells completely from medium and carried down less than 15 μl of medium as tested with [^{3}H]inulin. Cells were then dissolved in 0.4 M KOH–10% deoxycholate for determination of radioactivity and proteins [14].

2.3. *Quantitation of vitellogenin by immunoprecipitation*

When the amount of vitellogenin synthesized was to be assessed, cells from quadruplicate incubations (conditions for protein synthesis, see above) were pooled. The material dissolved in 0.4 M KOH–10% deoxycholate was extensively dialyzed against distilled water and finally against 0.15 M NaCl–0.01 M Tris, pH 7.5. After concentration of the solution with dextran T70 aliquots in triplicates were added to the reaction mixture containing antilipovitellin and processed according to Jost et al. [8]. The radioactivity in the immunprecipitate was compared to that obtained in the total protein precipitate and expressed as percentage.

2.4. *Determination of estradiol receptor*

This was done in purified nuclei according to our modification [15] of the original method of Mester and Baulieu [16].

3. Results

3.1. *Isolated hepatocytes take up estradiol and form estradiol-receptor complex*

As a prerequisite to investigate the effect of estradiol on protein synthesis, it was necessary to demonstrate that estradiol is actively taken up by isolated chick hepatocytes. Figure 1, panel A, demonstrates that such an uptake took place and was very rapid indeed, the maximum being measured at the earliest workable time point, e.g. 2 min. Hepatocytes metabolized estradiol in part which was evident by the increase in total labelled steroid (free and sulfated) in the surrounding medium. After 20 min plateau values were reached which remained constant up to 2 h (actual data not shown). In panel B only the CH_2Cl_2-extractable counts (free steroid) are plotted; free

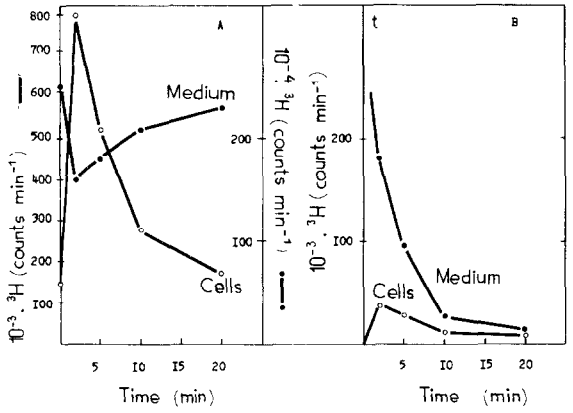

Fig.1. [^{3}H]estradiol uptake by isolated hepatocytes. Incubations of isolated hepatocytes were started by the addition of 2×10^6 cpm [^{3}H]estradiol and continued as described in the experimental section. At the times indicated total counts (panel A) and CH_2Cl_2-extractable counts (panel B) were determined in cells and medium.

estradiol disappeared rapidly from the medium to equilibrate with the cells within 20 min. These findings raised the question whether the steroid hormone was bound to a specific receptor protein. We tested this by incubating hepatocytes from untreated chicks under the standard conditions for 10 min with and without 10^{-6} M cold estradiol and determined quantitatively the high affinity ($K_D = 10^{-10}$ M) estradiol receptor in nuclei [21]. The results from two experiments (table 1) show that although a variable baseline

Table 1
Estradiol receptor content in nuclei of isolated chick hepatocytes

	Estradiol receptor (fmol/mg protein)		
	Control	Experimental	Δ%
Exp. 1	243	501	+206
Exp. 2	157	262	+167

Isolated hepatocytes from untreated chicks were incubated in quadruplicates for 10 min at 37°C with 10^{-6} M 17β-estradiol. The amount of incubation mixture and cells were doubled in each flask in order to have sufficient material available for nuclei preparation [6]. Nuclei were kept frozen at −80°C until quantitation of the hormone receptor. Hepatocytes incubated without estradiol were used as controls

of estradiol receptor existed, this was more than doubled within 10 min of incubation.

3.2. *Estradiol addition to perfusate leads to vitellogenin-synthesizing hepatocytes*

The above result led us to postulate that since in vivo the appearance of estradiol receptor [18] was followed in due time by the specific synthesis of vitellogenin [7], similar effects should be demonstrable in vitro. Thus the incorporation of [^{14}C]leucine into total and specific cellular proteins was studied in isolated hepatocytes incubated in vitro in the presence (E) or absence (C) of 10^{-6} M estradiol as a function of time. To these hepatocytes two types of primed cells were compared: hepatocytes from chicks which had been injected with estradiol 2½ days earlier (P) and hepatocytes isolated from livers perfused in the presence of estradiol for 60 min (A). Figure 2 shows that hepatocytes P incorporated [^{14}C]leucine linearly into proteins for at least 120 min at a rate which was much greater than that of hepatocytes A and also of hepatocytes C or E. Radioimmunprecipitation (table 2) demonstrated that hepatocytes P from chicks which in vivo actively synthesize vitellogenin [7] continued to do so in vitro for some time. After 2-h incubation the amount synthesized decreased however drastically. Hepatocytes A synthesized vitellogenin in vitro at an even greater rate than hepatocytes P but hepatocytes E were as unable as hepatocytes C to produce vitellogenin.

4. Discussion

Of the experiments with isolated hepatocytes reported here, two interesting findings emerge:

(1) The addition of estradiol to the incubation medium resulted in the appearance of nuclear estradiol receptor, but not in the synthesis of vitellogenin (table 1);

(2) The addition of estradiol to the perfusion medium or earlier, in form of injection to the animal, resulted in vitellogenin synthesis a few hours later (table 2, fig.2).

Concerning the first point, we wish to recall that in vivo estradiol produces within minutes the appearance of estradiol receptor complex in nuclei [15]. While this could be duplicated in vitro, the following event, i.e. the in vivo increase of RNA polymerase activities 2½ h after estradiol injection [17] did not occur (results not shown). Whether for this reason the hepatocytes failed to synthesize vitellogenin, we do not know at the moment. In vivo, the increased RNA polymerase activities are mediated through the estradiol receptor complex as we have shown in reconstitution experiments [18,19].

We could demonstrate that hepatocytes from liver

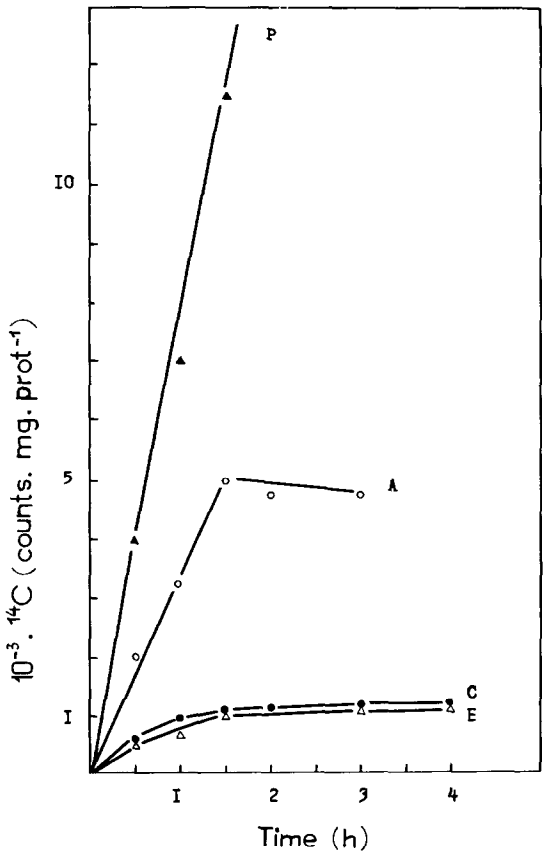

Fig. 2. Effect of estradiol on the incorporation of [^{14}C]leucine into cellular proteins of isolated hepatocytes. Isolated hepatocytes were incubated under conditions for protein synthesis (see experimental section) in the presence of 0.5 μCi [^{14}C]-leucine. Cellular radioactivity relative to protein content was determined at the times indicated. E, hepatocytes incubated in the presence of 10^{-6} M estradiol; C, controls; P, hepatocytes from chicks injected 2½ days earlier with estradiol and incubated in the absence of estradiol. A, hepatocyte from chicks which were perfused for 60 min in the presence of 1 mg estradiol.

Table 2
Effect of estradiol on vitellogenin synthesis in isolated hepatocytes

Type of hepatocytes	Incubation time	Incorporation of [^{14}C]leucine into		
	h	Total proteins (dpm/mg protein)	β_L-Lipovitellin (dpm/mg protein)	% of total proteins
A	2	19 230	100	0.52
A	4	13 278	103	0.77
P	2	19 719	144	0.73
P	3	29 940	184	0.61
P	4	815 859[a]	803	0.10
E	4	899 577[a]	174	0.02
C	4	754 770[a]	115	0.01

[a] In these experiments an additional 2.5 μCi of [^{14}C]leucine was added 60 min prior to the termination of the experiment

Isolated hepatocytes were from chicks injected with estradiol 2½ days earlier (P) or from livers perfused in the presence of estradiol for 60 min (A_1) or from controls incubated as such (C) or with estradiol (E). For details see section 2. Incubation was done in quadruplicate flasks containing the incubation mixture for protein synthesis including 0.5 μCi [^{14}C]leucine. The reaction was stopped by means of perchloric acid in special separation tubes and the cells were processed for immunoprecipitation with antilipovitellin as indicated in the experimental section

perfused in the presence of estradiol for 60 min synthesized vitellogenin in vitro 4 h later. We expected and showed that vitellogenin synthesis is maintained in vitro for a limited time if the hepatocytes had been synthesizing the protein in vivo already prior to their isolation. The failure to induce such a protein-synthesizing system by the mere addition of estradiol is not easily explained. Estradiol has been shown to enter the cells very rapidly and to be metabolized (fig.1), therefore failure of the hormone to enter the cells can be ruled out. We do not believe that the problem is one of difference in survival of hepatocytes from control chicks and of estrogen-treated chicks. It could be, however, a question of cell to cell contact which must occur at or during a specific time. In organ cultures [9,10,20] which synthesize vitellogenin cell to cell contact is maintained. Another possibility would be that serum needs to be present at a certain time providing a factor necessary in conjunction with estradiol. In support of this hypothesis is Carinci's work [21] who after 42 h withdrew serum containing estradiol from chick embryo liver cultures and the cells in an artificial medium continued to synthesize egg yolk protein in the absence of estradiol.

We feel that the experiments described here show a hopeful beginning for the use of the isolated hepatocyte in the study of egg yolk protein production and represent a contribution to the increasing knowledge concerning the biological conditions under which vitellogenin synthesis takes place.

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