ESTRADIOL RECEPTOR IN CHICKEN LIVER CYTOSOL

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1. Introduction

In oviparous vertebrates the liver is a target tissue for estradiol. The hormone induces the synthesis of several proteins among which is the major yolk protein precursor, vitellogenin [1]. From studies on other target tissues for estradiol, such as rat uterus [2] and chick oviduct [3], the existence of a high-affinity cytoplasmic receptor has emerged; this protein binds the hormone when it enters the cell. The hormone–receptor complex is transformed and subsequently translocated to the nucleus where the transformed complex is thought to regulate the transcription of certain genes by interacting with specific acceptor sites [2].

A high affinity estradiol-binding protein has been found in nuclei from livers of immature chickens, hens and roosters [4–8]. Estradiol administration increases the level of this receptor by ~10-fold. Nevertheless, either very small [10,11] or insignificant [4,5,8,9] concentrations were found when the same investigators were looking for a cytoplasmic estradiol receptor in liver. A transient increase of such a receptor was discovered [10] in liver from day 19 embryos and the number was of the right order to account for the rise in nuclear receptor after hormone administration. However, to our knowledge the tissue response to estradiol at this stage and after hatching is identical. Therefore we have to postulate either a mechanism of estradiol transport to the nucleus in the liver which differs from that in other target tissues, or to assume that the methods used for detection of the cytoplasmic receptor are less suitable for liver, except perhaps at a certain developmental stage.

We could show that the cytoplasmic estradiol receptor is present in the liver after hatching by devising an isolation method circumventing inactivation of the receptor and metabolism of the ligand. In immature chickens ⩾4000 specific, high-affinity binding sites/hepatocyte are present. The in vitro translocation of the hormone–receptor complex to the nucleus is also demonstrated.

2. Materials and methods

2.1. *Animals*

Hybrid chickens of Rhode Island Red × White Leghorn were used in this study. Fertilized eggs were bought from 'Hypeco' (Venray, The Netherlands) and kept in a humid incubator at 38°C. Male embryos at day 19 of incubation and week 5–10 roosters were used for the experiments.

2.2. *Materials*

The following materials were bought from the sources indicated: ultra pure ammonium sulfate (Schwarz-Mann); bovine serum albumine (Sigma); charcoal (Baker); Dextran 80 (Pharmacia); diethylstilbestrol (Sigma); heparin (Sigma); Nembutal (Abbott); protamine sulfate (BDH); salmon sperm DNA (Sigma); Sephadex G-50 (Pharmacia); Soluene-350 and Toluene Scintillator (Packard). [6,7-^{3}H]-Estradiol-17β (spec. act. 44 Ci/mmol) was purchased from New England Nuclear. Estradiol, estrone, estriol, testosterone and progesterone were generous gifts from Organon.

2.3. *Isolation of cytosol and purification of estradiol receptor*

Roosters were anaethesized with Nembutal (60 mg/kg body wt). Livers were excised after perfusion in situ with buffer A (50 mM Tris, 50 mM maleate, 1.5 mM EDTA, 1 mM dithiothreitol and 125 mM NaCl (pH 7.4 at 0°C)) to remove blood. When embryonic tissue was used the collected livers were extensively washed with buffer A. The tissue was weighed

and immediately minced in 4 vol. buffer B which had the same composition as the perfusion buffer A except that NaCl was replaced by KCl. Homogenization was done with a Dounce homogenizer (Kontes: pestle A 6 strokes and pestle B 3 strokes). The homogenate was centrifuged at 1500 × *g* for 15 min and the supernatant was centrifuged at 150 000 × *g* for 90 min. The lipid layer was carefully removed and the supernatant was used for further purification. Protamine sulfate (1 mg/ml) dissolved in buffer C, which was 5-fold diluted buffer B, and adjusted to pH 7.4 at 0°C, was slowly added under stirring to 0.55 mg/ml final conc. After 20 min, the precipitate was centrifuged at 700 × *g* for 10 min and washed with buffer C. The precipitate was solubilized in a heparin solution (6 mg/ml buffer C (pH 7.4 at 0°C)). Insoluble material was discarded by centrifugation at 10 000 × *g* for 10 min. To the supernatant a neutralized solution of saturated $(NH_4)_2SO_4$ was slowly added under stirring until a fractional saturation of 40% was obtained. After 15 min the suspension was centrifuged at 1500 × *g* for 15 min, the precipitate washed once with 40% $(NH_4)_2SO_4$ and finally centrifuged at 10 000 × *g* for 15 min. The precipitate was dissolved in a small volume of buffer B and insoluble material was removed by centrifugation (20 min at 10 000 × *g*). Sephadex G-25 chromatography in buffer B was used to free the solution of ammonium sulfate. The protein fraction obtained was diluted to half of its original cytosol volume with the same buffer. All steps were at 2°C.

2.4. *[^{3}H]Estradiol binding*

For binding 0.4 ml of the purified cytosol fraction was mixed with [^{3}H]estradiol and the assay mixture incubated for 16 h at 2°C. [^{3}H]Estradiol was 0.125–30 nM final conc. The binding was assayed by the protamine sulfate method [12] except that the precipitate was dissolved in Soluene-350 instead of extracting the bound radioactivity with alcohol. The dissolved precipitate was added to Toluene Scintillator and the radioactivity was measured in a Philips liquid scintillation counter with a counting efficiency of ~45%. To correct for non-specific binding parallel incubations were performed with [^{3}H]estradiol in the presence of 100-fold excess of unlabelled estradiol. The specific binding was calculated by subtracting non-specific binding from the total binding. The dissociation constants and the number of binding sites were determined by Scatchard analysis [13].

2.5. *Isolation of purified nuclei*

The liver was perfused as described above. The tissue was weighed, minced and homogenized in 9 vol. buffer D (0.32 M sucrose, 3 mM $MgCl_2$, 25 mM KCl, 50 mM Tris–HCl (pH 7.4 at 0°C)) with a Dounce homogenizer which was also used for resuspension of pellets in all subsequent steps (3 strokes of pestle B). The homogenate was passed through 3 layers of sterile gauze and centrifuged at 700 × *g* for 10 min at 2°C. The crude nuclear pellet was resuspended in buffer E (0.5 M sucrose, 2 mM $MgCl_2$, 25 mM KCl, 50 mM Tris–HCl (pH 7.4 at 0°C)) and centrifuged again at 700 × *g* for 10 min at 2°C. The nuclear pellet was resuspended in 9 vol. buffer E containing 2.0 M sucrose and 15 ml were layered on 15 ml buffer E with 2.0 M sucrose and centrifuged in a SW_{27} rotor at 25 000 × *g* for 20 min (0°C). The nuclear pellets were resuspended in buffer E in such a way that 1 ml contained the nuclei of 1 g tissue.

2.6. *Receptor binding to nuclei*

For the nuclear binding studies the receptor-containing cytosol was essentially purified as above except that the Sephadex G-25 chromatography was performed with a buffer of the following composition: 0.25 M sucrose, 25 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM Tris–HCl (pH 7.9 at 0°C). The receptor was charged with estradiol by incubation with 10 nM [^{3}H]estradiol overnight at 2°C. Excess hormone was removed by the addition of 1/10th vol. 5% charcoal 0.5% dextran suspension in buffer C. The mixture was vortexed and allowed to stand for 15 min with occasional shaking. Charcoal was removed by centrifugation for 15 min at 1500 × *g* (0°C). The nuclei pelleted in a polypropylene tube were resuspended in the [^{3}H]estradiol-charged cytosol fraction and incubated at the desired temperatures. After 30 min the nuclei were spun down at 700 × *g* for 10 min at 0°C and the hormone–receptor complex in the cytosol fraction was assayed by the charcoal–dextran method before and after incubation. Corrections for the stability of the complex were made by parallel incubations without nuclei. The nuclei were washed twice with 1 ml buffer E. Receptor bound to the nuclei was determined by extracting the pellets with 1 ml ethanol at room temperature for 30 min. The ethanol was evaporated at 50°C and the radioactivity was measured in 7 ml Toluene Scintillator.

2.7. *Thin-layer chromatography*

Hormone metabolites formed on incubation were analyzed by thin-layer chromatography according to [14]. Crude and purified cytosol fractions were incubated with 30 nM [^{3}H]estradiol for 16 h at 25°C and 10 μl aliquots were applied to ready-made thin-layer plates (DC Alufolien Kieselgel 60/Kiezelgur F_{254} plates from Merck). After chromatography the plates were dried, cut into pieces and the radioactivity was counted after adding 10 ml scintillation liquid.

2.8. *Protein and DNA determination*

Protein was measured by the Lowry method [15] with bovine serum albumin as a standard. DNA was estimated by the diphenylamine method as in [16] using salmon sperm DNA as standard.

3. Results and discussion

3.1. *The purification procedure*

Preliminary experiments showed that high-affinity binding sites could only be demonstrated in the cytosol from embryonic liver. After hatching significant amounts of cytosolic receptor could not be found, which is in agreement with the experience of other investigators. To resolve whether the cytosol from immature chicken liver contains factors degrading or masking the estradiol receptor we added 1 part of crude cytosol from livers of week 5 roosters to 2 parts of that prepared from embryonic liver. On incubation a substantial portion (80%) of the receptor appeared to have been lost. This loss is most likely due to receptor breakdown. However metabolism of [^{3}H]-estradiol might also contribute to the observed decrease in binding sites. In mammalian liver, rapid estradiol metabolism interferes with receptor assays [17]. We first tested whether cytosol from chicken liver also metabolizes estradiol. Fig.1A shows that >50% of the [^{3}H]estradiol added was metabolized and converted to estrone. This effect certainly contributes to the failure to detect the receptor in immature chicken liver. Moreover, we found that conversion of estradiol to estrone hardly occurs in embryonic liver cytosol (data not shown). To remove the metabolizing enzymes and other interfering factors, we developed a purification method based on properties of other estradiol receptors, mainly precipitation by protamine and ammonium sulfate. Fractionation of the cytosol by ammonium sulfate alone as was successfully applied for the mammalian liver [18] did not

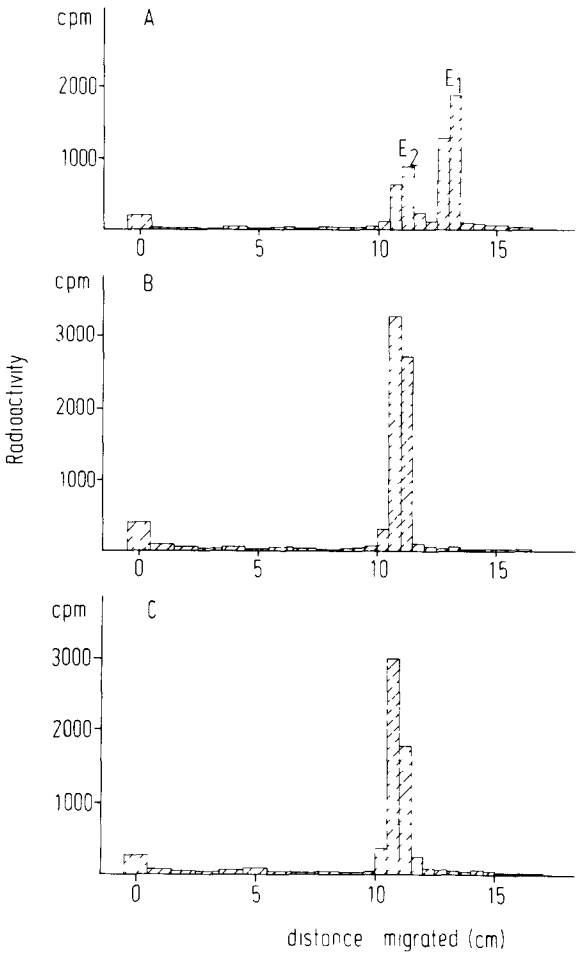

Fig.1. Analysis of [^{3}H]estradiol metabolism by thin-layer chromatography. Thin-layer chromatograms showing the distribution of radioactivity after incubation of the crude cytosol (A), and of the purified cytosol fraction (B), with 30 nM [^{3}H]estradiol for 16 h at 25°C. Both fractions were prepared from the same chicken. An incubation of [^{3}H]-estradiol in buffer was used as a control (C). Unlabelled hormones were used to identify the position of estradiol (E_2) and estrone (E_1).

result in the removal of enzyme activity. However, protamine sulfate precipitation turned out to be useful. Solubilisation of the protamine sulfate precipitate was achieved with a buffer containing heparin. The purification procedure, as detailed in section 2 resulted in a cytosol fraction which, as can be seen in fig.1B is virtually devoid of estradiol metabolizing activity and contains ~10% of the cytosolic protein. For the procedure we were guided by the estradiol-metabolizing enzymes. Fortunately this procedure also prevents, at

least to a large extent, the inactivation of the receptor, since now a significant number of binding sites is found.

3.2. *Properties of the receptor*

In the purified cytosol fraction binding sites for estradiol can be demonstrated. At 2°C equilibrium was reached after ~6.5 h; no loss in binding sites was found on further 18 h incubation. At higher temperatures even after short incubation, a decrease in binding sites was found. Fig.2 shows the estradiol binding and Scatchard plots for purified cytosol from livers of embryos at day 19 of incubation and day 70 roosters. It is obvious that in both preparations a single receptor is present with the same affinity; K_d 4×10^{-10} M. The concentration of binding sites calculated from the Scatchard plots is 104 and 83 fmol/mg protein for embryos and roosters, respectively. With embryonic tissue the recovery of our procedure is ~45%. Using this value and assuming that 1 g liver contains 2×10^8 hepatocytes [19] we calculated that there are 3500–4200 binding sites/hepatocyte present. Apparently during development no drastic changes in the binding activity for estradiol occur.

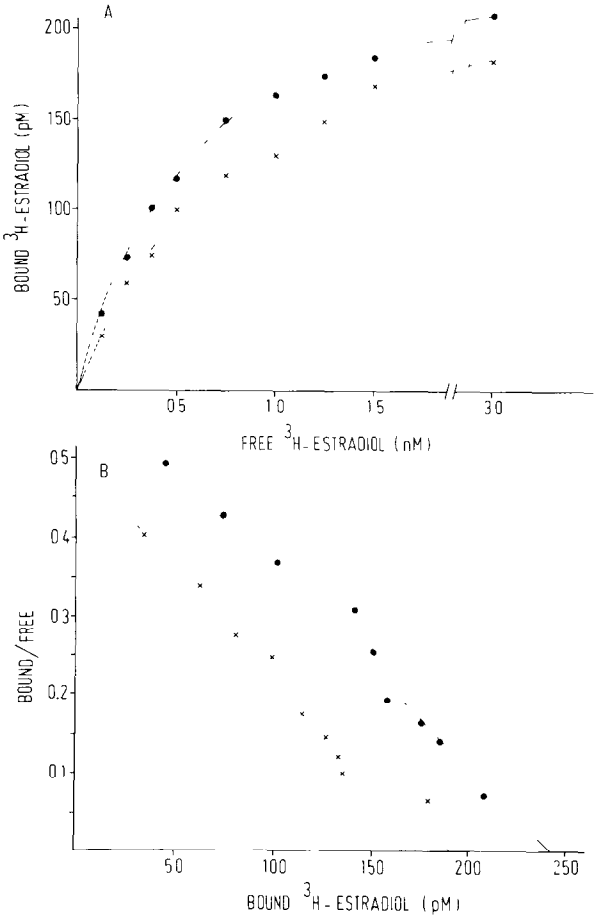

Fig.2. Determination of the dissociation constant and the number of binding sites. The [^{3}H]estradiol bound by the purified liver cytosol fraction was plotted for day 19 embryos (•–•) and for day 70 roosters (x-x) after incubation for 16 h at 2°C (A). Corrections for non-specific binding were made as in section 2. Scatchard plots were calculated from the binding curves (B). A purified cytosol fraction of 1 ml corresponds to the cytoplasm of 0.4 g liver tissue. The protein content was 2.3 mg/ml at both developmental stages.

The specificity of the binding with respect to various hormones is shown in table 1. The receptor has a high degree of specificity: only the estrogens compete with [^{3}H]estradiol for the binding site while testosterone, progesterone as well as cortisol are much less effective.

The binding activity is destroyed by trypsin but not by nucleases indicating its protein nature.

Whether the cytosolic receptor is taken up by nuclei was studied in vitro. Table 2 shows the uptake of the [^{3}H]estradiol–receptor complex by purified nuclei. An increase of the number of complexes in the nuclei and a concomitant decrease in the number of added cytosolic binding sites were observed. This process is temperature-dependent, as has been shown also for the uterus [2,20]. 20% of the specific binding sites added was taken up by the nuclei. Consequently, the concentration of the complex within the nuclei is ~8 times that in the surrounding cytosol.

Table 1
Competition of hormones for the estradiol binding site

Competitor	[^{3}H]Estradiol bound expressed as % control
Estradiol	1
Estrone	12
Estriol	19
Diethylstilbestrol	4
Testosterone	101
Progesterone	89
Cortisol	82

A sample of the purified cytosol fraction was incubated with 1.5 nM [^{3}H]estradiol and a 100-fold excess of a competitor hormone for 16 h at 2°C. The binding activity in these experiments was compared with an incubation in which no competitor was present. Corrections for non-specific binding were made as in section 2

Table 2
Uptake of the [^{3}H]estradiol–receptor complex by purified nuclei at two different temperatures

Temp. (°C)	Depletion of the complex in the cytosol (dpm)	Uptake of the complex by nuclei (dpm)
0	153	177
25	710	732
	714	812

The receptor in the purified cytosol fraction from livers of immature chicken was charged overnight at 2°C with 10 nM [^{3}H]estradiol. Excess of hormone was removed by the charcoal–dextran technique. Nuclei (0.24 mg DNA) were incubated with 0.4 ml charged cytosol fraction containing 3476 dpm specific bound [^{3}H]estradiol. Corrections for instability at 25°C were made

3.3. *Concluding remarks*

The number of binding sites determined is a minimum value, but is of the same order of magnitude as in other tissues, e.g., chicken oviduct. Moreover the number is sufficient to account for the fast increase in nuclear receptor sites after estradiol administration. Apparently liver forms no exception to the model of hormone–receptor translocation to the nucleus as proposed for other target tissues.

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