ESTROGEN BINDING SITES IN THE EMBRYONIC CHICKEN LIVER

Michael GSCHWENDT
Deutsches Krebsforschungszentrum, Institut für Biochemie, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG

Received 22 January 1977

1. Introduction

Estradiol induces and controls the synthesis of egg-yolk proteins in the liver of the laying hen as well as of male and immature birds (for review see refs [1,2]). Cytoplasmic and nuclear hormone-binding proteins (receptors) play an important role in steroid hormone mediated processes (for review see refs [3,4]). The chicken liver contains at least nuclear high affinity estrogen-binding proteins, which are in part soluble [5] and in part tightly sticking to the chromatin [6–9]. The small number of these nuclear receptor molecules in untreated animals is increased several-fold after estrogen treatment [8]. In the cytoplasm of the chicken liver only a low affinity estrogen-binding protein could be demonstrated up to now [10]. Therefore, it was of interest to look for estrogen receptors during the embryonic development of the chicken liver. The aim of this investigation was to induce nuclear estrogen-binding proteins in the embryonic liver by estrogen-treatment of eggs, to characterize these binding sites and to compare them to those from the liver of hatched animals.

2. Materials and methods

2.1. *Materials*

The following materials were obtained from the sources indicated: [6,7-$^3H_2$]Estradiol, spec. act. 48 Ci/mmol (New England Nuclear). Estradiol (1,3,5(10)-estriene-3,17β-diol), diethylstilbestrol (4,4-dihydroxy-*trans*-7,7′-diethylstilbene) and cortisol (11β,17α,21-trihydroxy-4-pregnene-3,20-dione) (Merck, Darmstadt). Progesterone (4-pregnene-3,20-dione) (Serva, Heidelberg). Dihydrotestosterone (17β-hydroxy-androstan-3-one) (Schering AG, Berlin). Bovine serum albumin, myoglobin and ovalbumin (Serva, Heidelberg).

2.2. *Animals*

Preincubated eggs were obtained from a local hatchery and kept in a humid incubator at 38°C. The eggs were injected with 0.1 ml of various solutions of hormones in propylene glycol as indicated in the text or with propylene glycol alone (control). After the injection the small hole in the shell was closed with a piece of adhesive tape and the eggs were again kept in the incubator for various times as indicated in the text.

2.3. *Isolation and extraction of crude and pure nuclei*

The livers from 10 embryos were combined. A crude nuclear preparation was obtained from the livers and its extraction was carried out essentially as described by Mester and Baulieu [5] with three modifications:

(i) 1 mM Dithioerythrol was added to all buffers.

(ii) The extraction-buffer (DETK) contained 1 mM dithioerythrol, 1 mM EDTA, 1 M KCl and 10 mM Tris–HCl, pH 7,4.

(iii) The nuclear extract was centrifuged for 4 h at 150 000 × *g* to completely remove all the chromatin.

The isolation of purified nuclei was performed as described earlier [8] and their extraction was carried out as described for crude nuclei. The nuclear extracts were then preincubated with 1/10 vol. of a dextran/charcoal suspension in DETK (2.5% Norit A, 0.25% dextran (M_r 80 000, Pharmacia)) at 45°C for 10 min to remove bound estradiol from the receptor.

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2.4. *Measurement of high affinity estrogen binding*

Two hundred μl of a nuclear extract were incubated at 5°C for 18 h with 20 μl [^{3}H]estradiol in DETK (0.625, 1.25, 2.5 and 5 nM final concentration) and 20 μl DETK or 20 μl of estradiol in DETK (a 100-fold excess over the concentration of [^{3}H]estradiol). The incubation was stopped by the addition of 200 μl of a dextran/charcoal suspension in DETK (0.25% Norit A, 0.025% dextran). The mixture was kept at 0°C for 30 min and was then centrifuged at 1000 X *g* for 10 min. 200 μl of the supernatant were used for the measurement of the radioactivity. Values obtained from incubations with the addition of a 100-fold excess of unlabelled estradiol (low affinity binding) were subtracted from those obtained without the addition of unlabelled estradiol. The result gives the amount of high affinity binding. Unbound radioactivity was calculated by subtracting bound radioactivity (high affinity binding) from the total radioactivity in the incubation mixture. Bound [^{3}H]estradiol (nM) and the radio bound/unbound [^{3}H]estradiol were then plotted according to the method of Scatchard [11]. The negative inverse of the slope of the straight line (see fig.4b) is the K_d and the intersection with the abscissa is the total number of binding sites in the incubation mixture, which will be given as fmol/mg protein.

2.5. *Measurement of radioactivity*

As described previously [9].

2.6. *Determination of protein in the nuclear extracts*

Five ml trichloroacetic acid (TCA) were added to 200 μl of a nuclear extract. The mixture was kept at room temperature for 20 min and filtered through glass-fibre filters (GF/C, Whatman). Each filter was washed two times with 5 ml TCA, dried and solubilized in 10 ml 1 N NaOH. After a short centrifugation to remove the filter residues, 200 μl of the supernatant were used for the determination of protein according to the method of Lowry [12]. Bovine serum albumin was used as a standard and treated the same way as the samples. This procedure was used to remove the buffer components from the nuclear extract, especially dithioerythrol, which interferes with the protein test.

2.7. *Sucrose-gradients*

Nuclear extracts were incubated with 5 nM [^{3}H]estradiol at 5°C for 18 h and unbound [^{3}H]estradiol was removed as described in section 2.4. Two hundred μl of a sample were layered on approx. 3.5 ml of a SW60 rotor (Beckman) at 55 000 rev./min for 18 h. Myoglobin, ovalbumin and bovine serum albumin were used as internal as well as external standards. By use of an ISCO density-gradient fractionator, fractions (10 drops, approx. 0.18 ml each) were collected directly into scintillation vials and the radioactivity was measured as described previously [9].

3. Results

The liver of 15 day-old chicken embryos contains nuclear estrogen receptor sites, which are increased several-fold after the injection of estradiol into the egg. This increase is dependent on the dose of estradiol (fig.1). A maximum stimulation of the amount

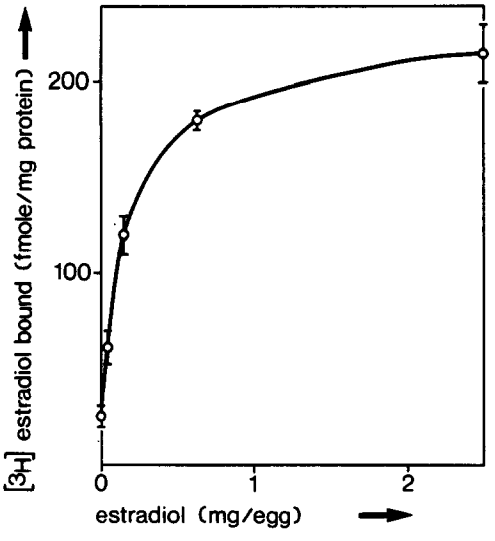

Fig.1. Dose-dependence of the estradiol-induced increase of estrogen-binding sites in the liver of 15 day-old chicken embryos. Eggs were injected with various amounts of estradiol dissolved in 0.1 ml propylene glycol. 24 h later the livers were excised from the embryos. 10 Livers were combined, a crude nuclear extract was prepared and incubated with increasing amounts of [^{3}H]estradiol as described in Materials and methods. The results were plotted according to the method of Scatchard and the number of binding sites (fmol/mg protein) estimated as described in Materials and methods. Each value is the mean of two experiments.

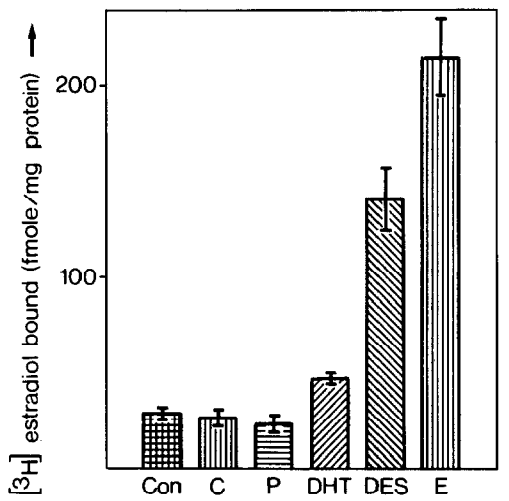

Fig.2. Hormone-dependence of the increase of estrogen binding sites in the liver of 15 day-old chicken embryos. Eggs were injected with various hormones (2.5 mg) dissolved in 0.1 ml propylene glycol. The remaining experimental details were as described in fig.1. Con = control, C = cortisol, P = progesterone, DHT = dihydrotestosterone, DES = diethylstilbestrol, E = estradiol.

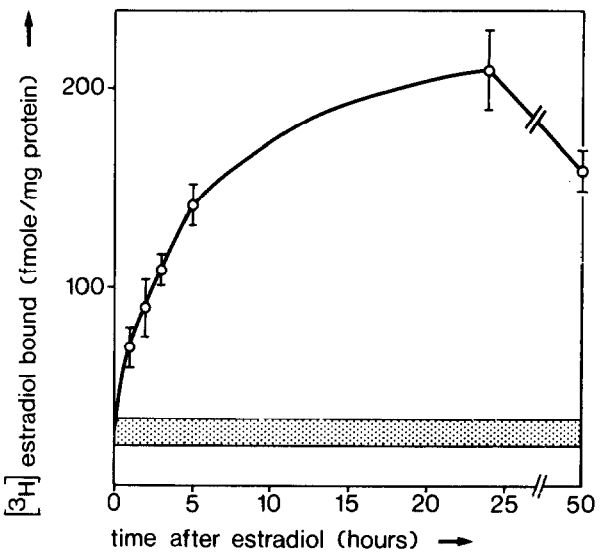

Fig.3. Time-dependence of the estradiol-induced increase of estrogen binding sites in the liver of 15 day-old chicken embryos. Eggs were injected with 2.5 mg estradiol in 0.1 ml propylene glycol. Various times later, the livers were excised from the embryos. Other details of the procedure were as described in fig.1. The hatched area represents the number of binding sites in untreated livers.

of binding sites is observed with 2.5 mg estradiol/egg. The portion of the injected estradiol, which actually gets into the liver of the embryo, however, is not known. This will be the subject of further investigations.

Several hormones were tested for their ability to raise the level of the nuclear estrogen receptor content in the embryonic liver (fig.2). Estradiol itself is the most potent inducer, followed by diethylstilbestrol, which seems to be somewhat less effective. Dihydrotestosterone appears to be a very weak inducer, whereas progesterone and cortisol do not produce any effect.

Figure 3 shows the increase of estrogen-binding sites dependent on the time after the injection of estradiol into the eggs. As soon as one hour after the injection of estradiol, the shortest time interval determined, a significant (2- to 3-fold) increase of receptor sites can be observed. This increase continues up to about 15–24 h after the injection of estradiol, where a plateau with a maximum increase of 6–8-times of the control value is reached. Fifty hours after the injection of estradiol the amount of binding sites has started to decrease again.

The KCl-soluble nuclear estrogen-binding sites of the estrogenized embryonic chicken liver exhibit a high affinity for estradiol. They can be saturated with estradiol in the nM-range (fig.4a). The dissociation constant (K_d) of the estradiol–receptor complex estimated from a Scatchard plot (fig.4b) is $1-3 \times 10^{-10}$ M. 210 ± 30 fmol estrogen-binding sites/mg protein are found in a crude nuclear extract, whereas an extract from purified nuclei (not shown) contains 780 ± 25 fmol/mg protein. The number of binding sites/mg DNA, however, is about the same for nuclear extracts from crude and pure nuclei (~700 fmol/mg DNA). About 70% of the total nuclear binding sites can be extracted from purified nuclei by 1 M KCl.

On a 5–20% sucrose-gradient (fig.5) the estrogen receptor from a crude nuclear extract behaves similar to ovalbumin with a sedimentation coefficient of about 3.8 S, whereas the estrogen-binding sites of an extract from purified nuclei tend to aggregate and sediment to the bottom of the tube. A similar behaviour has been observed with pure nuclear

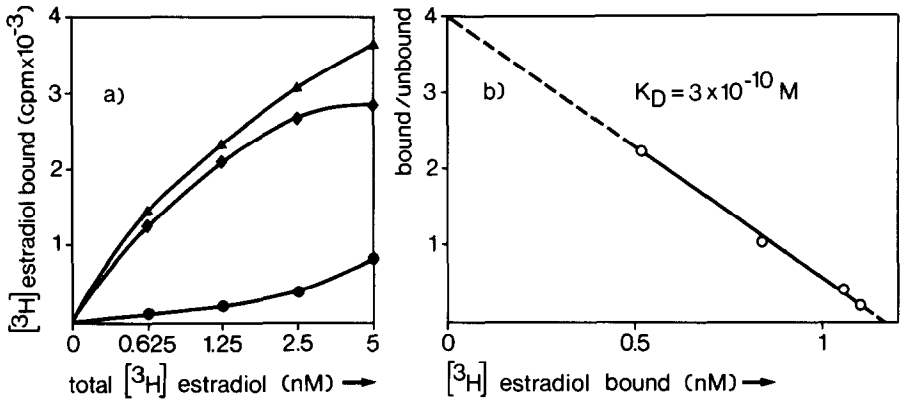

Fig.4. High-affinity binding of [^{3}H]estradiol to the nuclear binding sites of embryonic chicken liver. The procedure was as described in Materials and methods and in the legend to fig.1. Fig.4a. (●——●) Low-affinity binding, (▲——▲) total binding, (♦——♦) high-affinity binding (total minus low-affinity). Fig.4b. The high-affinity binding (see fig.4a) was plotted according to the method of Scatchard as described in Materials and methods.

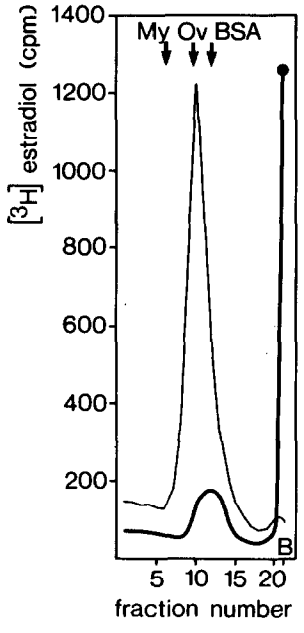

Fig.5. Sedimentation-profiles of nuclear extracts from crude and purified nuclei. The procedure was as described in Materials and methods. After the fractionation of the gradients the radioactivity in the dissected bottom of the tube (B) was determined separately. Myoglobin (My, 2.04 S), ovalbumin (Ov, 3.67 S) and bovine serum albumin (BSA, 4.5 S) were used as standards and were layered either together with the nuclear extract on a gradient or on separate gradients. The arrows indicate the maximum absorption of the protein standards at 280 nm. (———) Nuclear extract from crude nuclei. (▬▬) Nuclear extract from purified nuclei.

extracts from estrogenized immature chicken liver and will be reported elsewhere (W. Schneider and M. Gschwendt, in preparation).

4. Discussion

Estradiol can be easily injected into eggs. At least at an age of 10 days (the earliest stage of development investigated) and later (for most experiments 15 day-old embryos were used) the nuclear estrogen receptor content of the embryonic liver is increased by such a single injection of estradiol. This increase of nuclear receptor sites is dose-dependent and specific for estrogenic hormones. These properties including the affinity of the nuclear receptor for estradiol, the number of binding sites and the behaviour on sucrose-gradients are very similar to those found for the nuclear estrogen receptor from immature and adult chicken liver ([5], W. Schneider and M. Gschwendt, in preparation).

Part of the nuclear estrogen receptor from embryonic chicken liver (about 30%) remains tightly bound to the chromatin. It can be solubilized in 2 M KCl/5 M urea and fractionated on hydroxylapatite (M. Gschwendt, unpublished results) as described for the liver of immature animals [9].

These data indicate that embryonic chicken liver can produce the nuclear estrogen receptor and

is sensitive to estradiol in the same way as the liver of hatched animals, at least with respect to an increased formation of nuclear receptor sites. This is in accordance with results reported on other estrogen target-tissues like the uterus [13] or the chicken oviduct [14], where the presence of the respective receptors could be demonstrated at various embryonic stages. If the embryonic chicken liver is sensitive to estradiol also with respect to the induction of the synthesis of egg-yolk proteins, remains to be investigated.

Preliminary results indicate that a small number of cytoplasmic high affinity estrogen-binding sites ($K_d \sim 10^{-9}$ M) can be demonstrated in the embryonic chicken liver. This is in contrast to results obtained with the liver of immature and adult animals, where the high affinity estrogen receptor might be masked by a large amount of low affinity binding sites [10]. Experiments are under way to characterize this cytoplasmic receptor.

Acknowledgements

Mr Walter Kittstein provided excellent technical assistance.

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