RAPID APPEARANCE OF ESTROGEN RECEPTOR IN CHICK LIVER NUCLEI: PARTIAL INHIBITION BY CYCLOHEXIMIDE

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1. Introduction

Estrogens and other steroid hormones initiate their action by first binding to a specific cytoplasmic protein, the receptor; the resulting complex is then translocated to the nucleus where the hormone receptor complex binds to specific acceptor sites on the chromatin. It is assumed that the cytoplasmic receptor and the nuclear receptor are one and the same protein or possess at least a precursor–product relationship although no clear-cut demonstration exists as yet. Thus, disappearance of cytoplasmic estrogen receptor in rat uterus [1–3] was followed by appearance of nuclear receptor within 30 min after hormone injection [3,4]. Similar data have been published for chick oviduct [5,6]. Cytosol as well as nuclei from rat liver contain estradiol binding proteins [7], so does chick liver chromatin [8] and supposedly also chick liver cytosol [9] although the latter data could not be confirmed [8,10,11] nor have we ever found receptor activity in chick liver cytoplasm. Nevertheless, to get information on a possible very rapid translocation in this particular target tissue of a cytoplasmic receptor molecule to the nucleus, we approached the problem as follows. Using an intravenous injection route leading directly to the liver – described in this paper – we were able to immediately bring estradiol in contact with the target organ and thus to determine as a function of time the appearance of nuclear estradiol receptor. Indeed, within one minute we could already show half maximal nuclear receptor activity; full activity being attained within 10 min. In spite of lack of evidence for a cytoplasmic receptor, this does not exclude an extremely rapid translocation. Further, we show here that cycloheximide given in vivo partly prevents this appearance of receptor activity which suggests either de novo synthesis of the receptor molecule, or its subunits, or the possible involvement of a polypeptide necessary for the association of the nuclear receptor with the acceptor sites on the chromatin.

Lastly, following a single injection of estradiol, we studied the estrogen receptor in chick liver nuclei over a period of several days and found that it disappeared so slowly that after a week levels were still greater than those of the controls. This may be why a second injection of estradiol given at this time produces vitellogenin faster and in larger amounts [12,13].

The facts that estradiol receptor can be shown to appear already within minutes in chick liver nuclei after in vivo estrogen treatment and that increases in nuclear RNA [14], synthesis of specific nuclear non-histone proteins [15] and activities of DNA dependent RNA polymerases occur within hours [16,17] support the idea that the appearance of estrogen receptor may be the key event upon which depend all further biochemical steps leading to the synthesis of vitellogenin.

2. Experimental

2.1. *Animals*

One week old white leghorn chicks of both sexes were obtained locally and maintained in electrically heated brooders. Chicks of either sex were used when they had reached a minimum body weight of 100 g,

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after an overnight fast. They were injected with the hormone by two different routes: (a) intravenously: 17β-estradiol was dissolved in the quantities stated in an emulsion of sesame oil with phosphate buffered saline (Dulbecco's PBS). The emulsion was prepared according to Schurr [18] with a modification: a mixture consisting of 2.2 ml of sesame oil and 0.3 g of egg lecithin was homogenized at 60°C under a stream of nitrogen in a teflonglass homogenizer with 7.5 ml of a solution containing 0.3% of pluronic F-68 in PBS. Control animals received emulsion only. Birds were anaesthetized with nembutal (1.2 ml/kg) and the vena pancreatica duodenalis exposed surgically. Injections were done into this vein which leads directly into the portal vein. Sham operations with nembutal only did not affect the estradiol receptor level in any way. (b) Subcutaneously: 17β-estradiol (20 mg/kg body weight) was dissolved in 0.1 ml propylene glycol and injected under the skin of the leg. Control animals received propylene glycol only.

In preliminary studies, optimum conditions regarding injected 17β-estradiol concentration, weight of animals, etc., as a function of nuclear receptor activity were established. These demonstrated that birds up to 200 g body weight gave maximum receptor activities of approximately 150 fmol/mg protein when injected subcutaneously with a minimum of 10 mg estradiol/kg body weight. Routinely, birds weighing up to 200 g were used and injected with 20 mg estradiol/kg body weight.

2.2. *Preparation of nuclei and high salt extraction of estradiol receptor*

Birds were killed and the livers immediately cooled by perfusion of ice-cold PBS through the vena cava caudalis. Livers were then homogenized in three volumes of buffer A (0.32 M sucrose, 2 mM KH_2PO_4, pH 5.8, 3 mM $MgCl_2$, 0.5 mM mercaptoethanol) with 7 strokes in a Teflon-glass homogenizer at 2000 rev/min. The homogenate was layered over an equal volume of the above buffer made in 0.6 M sucrose and centrifuged at 400 *g* for 10 min. The purified nuclei were resuspended in buffer E (0.5 M KCl, 10 mM Tris, pH 8.0, 1 mM EDTA, 5 mM $CaCl_2$), frozen, thawed and centrifuged at 100 000 *g* for 45 min. This supernatant fluid contained the high-salt extractable hormone receptor complex which was further purified through an ammonium sulfate precipitation step. For this the supernatant fluid was adjusted to a final concentration of 3–5 mg protein/ml with buffer E and a saturated solution of ammonium sulfate in 100 mM Tris, pH 8.0, was added under a stream of nitrogen to 42% saturation. The resulting precipitate was centrifuged at 20 000 *g* for 10 min and the pellet was dissolved in buffer E followed by a second centrifugation at 20 000 *g* for 30 min. This supernatant fraction was quantitatively assayed for receptor activity as follows.

2.3. *Determination of estradiol receptor*

The method of Mester and Baulieu [11] was used with some modifications: To 3 ml of supernatant fluid obtained after the ammonium sulfate step, containing up to 4 mg protein/ml, we added 0.7 ml of buffer E containing in addition 0.5% charcoal and 0.005% dextran T 70 (buffer F) and incubated this mixture for 30 min at 37°C. The charcoal was removed by centrifugation. This preincubation procedure quantitatively removes the endogenous estradiol [11]. For the binding assay, aliquots of 300 μl of the charcoal-treated extract were incubated at 2°C for 2 h with varying concentrations of [^{3}H]estradiol in the range of 10^{-8}–10^{-10} M in a total volume of 0.5 ml buffer E. The reaction was terminated by the addition of 100 μl buffer F and further incubation at 2°C for 30 min followed by removal of the charcoal by centrifugation. A 0.5 ml portion of the supernatant fluid (bound radioactivity) and also of the [^{3}H]estradiol (total radioactivity) were counted in a scintillation cocktail consisting of 1% butyl PBD in a mixture of Triton X-100 and xylene (3 : 7) using an Isocap liquid scintillation counter. The results obtained were plotted according to Scatchard (19). A typical .plot. is shown in fig.1.

2.4. *Chemicals*

Butyl-PBD and 17β-estradiol were both obtained from CIBA-GEIGY Limited, Basel; pluronic F-68 was from Wyandotte Chemical Corp., Wyandotte, Michigan, USA; charcoal (norite A) was bought from Serva Ltd., Heidelberg, Germany, and dextran T 70 from Pharmacia, Uppsala, Sweden. Cycloheximide was from Calbiochem. New England Nuclear was the supplier for [6,7-^{3}H(N)]estradiol-17β (spec.act. 48 Ci/mmol). All other chemicals were of analytical grade.

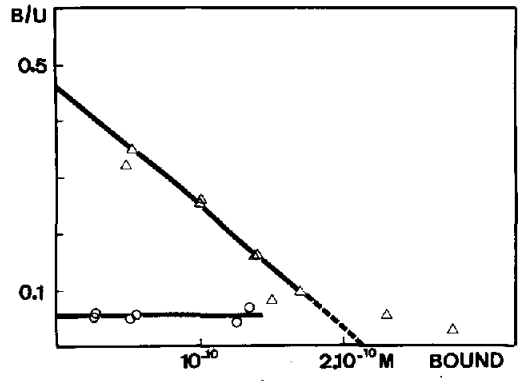

Fig.1. Scatchard analysis of the binding of [^{3}H]estradiol by high-affinity sites on the receptor. Experimental details are described in the text. Bound estradiol (B) was determined directly as dpm and unbound estradiol (U) was calculated from the total of estradiol added. No unlabelled estradiol added: (△——△). From the slope the calculated dissociation constant, $K_D = 4.4 \times 10^{10}$ M and from the intercept on the abscissa, the concentration of binding sites = 2.1×10^{-10} M are obtained. If a 100-fold excess of unlabelled estradiol is present during the incubation, a horizontal line is obtained (○——○).

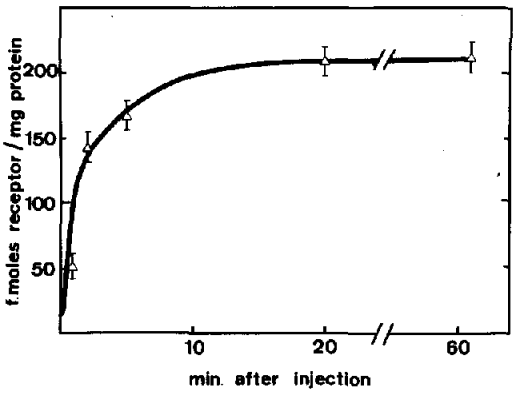

Fig.2. Kinetics of appearance of nuclear estrogen receptor after portal vein injection of estradiol. Groups of anaesthetized chicks (130 ± 20 g body weight) were injected with estradiol (50 μg/kg) into the portal vein. Chicks were sacrificed at various times after injection as indicated in the graph and the hormone receptor was assayed as described in the experimental section.

3. Results

3.1. *Rapid appearance of receptor after intravenous injection*

The appearance of the estrogen receptor after injecting estradiol directly into the portal vein was extremely rapid. In less than 1 min, the receptor concentration had risen to four times the base level (fig.2) and within 10 min it had reached its maximum. This could be achieved with as little as 2 μg estradiol/kg only if the injection site was the portal vein. Wing vein injections required at least 10 times as much estradiol to obtain maximum receptor levels because of the dilution of the hormone when it circulates throughout the body before reaching the liver. The delay in appearance, however, was negligible (actual data not shown). Injections of physiological quantities of estradiol of the order of 50 pg/ml [20] did not result in measurable increases of nuclear estrogen receptor.

3.2. *Effect of inhibition of protein synthesis on the appearance of the receptor*

In a preliminary test we established the efficiency of cycloheximide to inhibit protein synthesis. Chicks received 1 mg cycloheximide/kg into the wing vein and 5 mg/kg intraperitoneally immediately thereafter. This led to > 95% inhibition of [^{3}H]leucine incorporation into liver proteins for the period 5–120 min after injection (table 1). Groups of three chicks were given 5 min pulses of 50 μg estradiol/kg into the wing vein at various times after injection of cycloheximide as above (fig.3). We observed an almost

Table 1
Inhibition of protein synthesis in chick liver after in vivo cycloheximide treatment

Time after cycloheximide (min)	[^{14}C]Amino acid incorporation (dpm/mg protein)
0	3360 ± 400
5	115 ± 20
120	50 ± 10

Groups of 3 chicks (130 ± 10 g) were injected with 1 mg/kg cycloheximide into the wing vein at time 0, followed by 5 mg/kg cycloheximide intraperitoneally. 10 min before the indicated times, a 10 min pulse of 200 μl [^{14}C]amino acid mixture (50 μCi/ml, Radiochemical Centre, Amersham) was given into the wing vein. Controls received the amino acid pulse only. Chicks were sacrificed, their liver excised and the amino acid incorporation into total proteins determined according to Jost et al. [16].

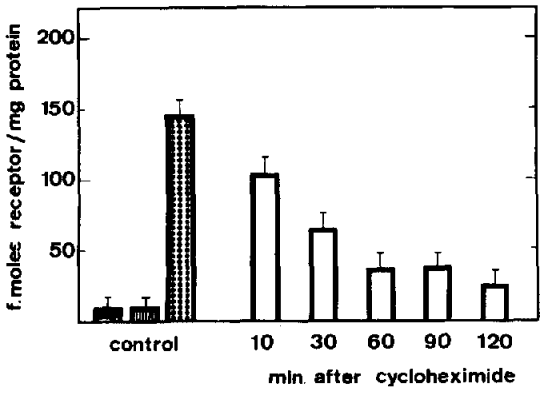

Fig.3. Effect of inhibition of protein synthesis on the appearance of the nuclear receptor. Chicks (130 ± 20 g body weight) were injected with 1 mg cycloheximide/kg into the wing vein followed by 5 mg/kg cycloheximide intraperitoneally. The estrogen receptor was induced by a 5 min pulse of estradiol (50 μg/kg) given into the wing vein at varying times after cycloheximide treatment (empty bars). Control chicks received no treatment (horizonally lined bars), cycloheximide only (vertically lined bars), or estradiol only dotted bars.

normal appearance of hormone receptor with an estradiol pulse beginning 10 min after cycloheximide administration, the response then gradually diminished when estradiol was given 30 min, 60 min and 90 min after cycloheximide. Surprisingly, the receptor response never disappeared completely and receptor levels of twice the controls value remained, even 2 h after cycloheximide treatment.

3.3. *Long-term kinetics of nuclear estrogen-receptor concentration as a function of estradiol*

For these studies estradiol was injected subcutaneously. Fig.4A shows the initial rapid increase of nuclear receptor and its slow disappearance over days concomitantly with the disappearance of the hormone from the blood (fig.4B). This figure also shows that at the site of injection a depot of estradiol is formed which diminishes very rapidly during the first 24 h, then much more slowly and steadily for more than a week. The rate of appearance of the nuclear receptor, measured in crude extracts, was biphasic: there was a

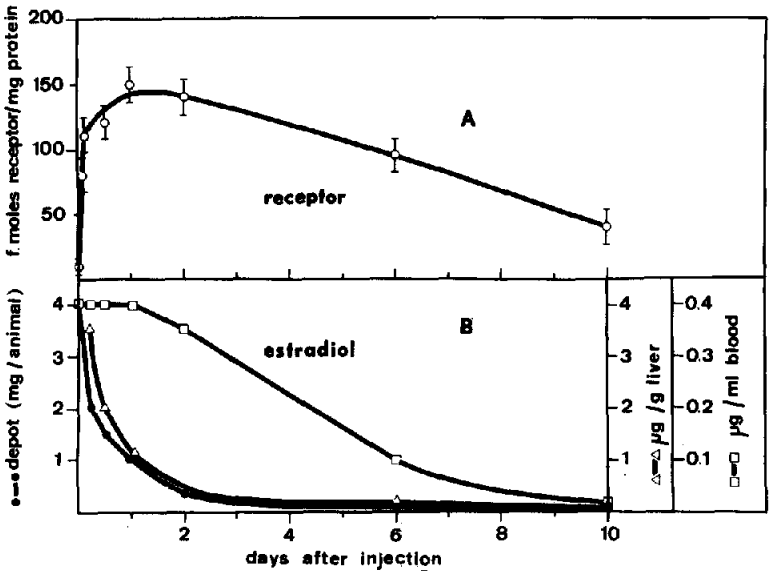

Fig.4. Long-term kinetics of estrogen receptor as a function of estradiol concentration. 4 mg [^{3}H]estradiol (2 × 10^6 dpm/mg) dissolved in propylene glycol was injected subcutaneously into chicks (200 ± 20 g body weight). Chicks were sacrificed at the times indicated and [^{3}H]estradiol was counted after combustion in an oxidizer (model Oximat, Intertechnique) in portions of skin taken from the injection site (•——•), in portions of liver (△——△) and of blood (□——□). In parallel, an equal group of chicks received 20 mg/kg estradiol subcutaneously. From these birds nuclear extracts were prepared at the times indicated and the estrogen receptor assayed as described.

very fast increase within the first hour to a level of 70–100 fmol/mg protein followed by a slower increase within the next 6–20 h to a peak level of 140 fmol/mg protein.

4. Discussion

Our present data show 3 facts: (a) estrogen receptor appearance in chick liver nuclei in a matter of minutes, (b) inhibition of protein synthesis in part inhibits appearance of estrogen receptor and (c) our long-term data show that estrogen receptor activity still persists after 7 days, a time at which vitellogenin synthesis has ceased [12]. The last finding may be of prime importance because it is known that a secondary stimulation of chicks by estradiol results in a much faster and greater production of vitellogenin [12,13].

A level of approximately 1000 high-affinity estradiol binding sites/cell ($K_D = 10^{-9}-10^{-10}$ M) has been determined from studies of liver chromatin from roosters pretreated with 17β-estradiol for periods of 15 min to 24 h [21]. Our results confirm these data of Gschwendt [8,21] and extend the study in the sense that we were able to determine receptor activity as soon as 1 min after hormone treatment. One can calculate that in our control animals there were approximately 140 sites/cell in good agreement with the data of Lebeau et al. [22] who found 100 such sites/cell in liver nuclei from untreated animals; this number was increased 3–5 times in laying hens or 24 h estrogen-treated chicks. These figures were arrived at by combining a high-salt extraction method with a trypsin method while we used only a high-salt method but in combination with freezing and thawing.

Gschwendt [8] also demonstrated that the estradiol binding capacity of rooster liver chromatin prepared from animals which had received cycloheximide was decreased by 60% within 30 min. We have confirmed this and we should like to suggest that a rapidly turning over polypeptide is most probably a crucial factor in the estrogen binding property of the receptor molecule. This could be one of the subunits of the receptor itself [23] or a polypeptide involved in binding the hormone receptor complex to the acceptor site. That chromosomal proteins are involved was shown by Gschwendt [21] who was unable to find a binding capacity after pronase treatment of chromatin.

Estrogen receptor binding to its acceptor site may well be the event that triggers off subsequent biochemical sequences. We have recently shown by reconstitution experiments [24] that estrogen receptor complex activates RNA polymerases I and II in chick liver chromatin. Such an activation – of the nucleolar RNA polymerase I in calf uterus – had already been proposed by Arbaud et al. in 1971 [25,26] and an association of RNA polymerase I with estrogen receptor in quail oviduct evident during purification was demonstrated by Müller et al. [27]. In conclusion, the increased number of high affinity binding sites for estradiol found in target cells occurring extremely rapidly after in vivo hormone treatment and disappearing extremely slowly may well be the prerequisite biochemical event which precedes changes in genetic transcriptional activity.

References

[1] Toft, D. and Gorski, J. (1966) Proc. Natl. Acad. Sci. US 55, 1574–1581.
[2] Jensen, E. V. and DeSombre, E. R. (1973) Science 182, 126–134.
[3] O'Malley, B. W. and Means, A. R. (1974) Science 183, 610–620.
[4] Mester, J. and Baulieu, E. E. (1975) Biochem. J. 146, 617–623.
[5] Cox, R. F., Catlin, G. H. and Carey, N. H. (1971) Eur. J. Biochem. 22, 46–56.
[6] Kalimi, M., Tsai, S. Y., Tsai, M.-J., Clark, J. H. and O'Malley, B. W. (1976) J. Biol. Chem. 251, 516–523.
[7] Eisenfeld, A. J., Aten, R., Weinberger, M., Haselbacher, G., Halpern, K. and Krakoff, L. (1976) Science 191, 862–865.
[8] Gschwendt, M. and Kittstein, W. (1974) Biochim. Biophys. Acta 361, 84–96.
[9] Arias, F. and Warren, J. C. (1971) Biochim. Biophys. Acta 230, 550–559.
[10] Ozon, R. and Bellé, R. (1973) Biochim. Biophys. Acta 297, 155–163.
[11] Mester, J. and Baulieu, E. E. (1972) Biochim. Biophys. Acta 261, 236–244.
[12] Jost, J.-P., Keller, R. and Dierks-Ventling, C. (1973) J. Biol. Chem. 248, 5262–5266.
[13] Deeley, R. G., Mullinix, K. P., Wetekam, W., Kronenberg, H. M., Mayers, M., Eldridge, J. D. and Goldberger, R. F. (1975) J. Biol. Chem. 250, 9060–9066.
[14] Dierks-Ventling, C. and Jost, J.-P. (1974) J. Cell Biol. 63, 84a.

[15] Dierks-Ventling, C. and Jost, J.-P. (1974) Eur. J. Biochem. 50, 33–40.
[16] Bieri, F. and Dierks-Ventling, C. (1975) Experientia 31, 734.
[17] Van den Berg, J. A., Kooistra, T., Ab, G. and Gruber, M. (1974) Biochem. Biophys. Res. Comm. 61, 367–374.
[18] Schurr, P. E. (1969) Cancer Res. 29, 258–260.
[19] Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672.
[20] Schröcknadel, H., Bator, A. and Frick, J. (1973) Steroids 22, 767–773.
[21] Geschwendt, M. and Kittstein, W. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 1642–1644.
[22] Lebeau, M. C., Massol, N. and Baulieu, E. E. (1974) FEBS Lett. 43, 107–111.
[23] O'Malley, B. W. (1976) ICN-UCLA Symposium on Molecular Mechanisms in the Control of Gene Expression, in press.
[24] Dierks-Ventling, C. and Bieri-Bonniot, F. (1976) ICN-UCLA Symposium on Molecular Mechanisms in the Control of Gene Expression, in press.
[25] Arnaud, M., Beziat, Y., Guilleux, J. C., Hough, A., Hough, D. and Mousseron-Canet, M. (1971) Biochim. Biophys. Acta 232, 117–131.
[26] Arnaud, M., Beziat, Y., Borgna, J. L., Guilleux, J. C. and Mousseron-Canet, M. (1971) Biochim. Biophys. Acta 254, 241–254.
[27] Müller, W. E. G., Totsuka, A. and Zahn, R. K. (1974) Biochim. Biophys. Acta 366, 224–233.