Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 1039–1043. Pergamon Press. Printed in Great Britain

# UTERINE RECEPTOR–ESTRADIOL COMPLEXES AND THEIR INTERACTION WITH NUCLEAR BINDING SITES

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## SUMMARY

Extensive analysis of nuclear bound estrogen receptor complexes ($R_nE$) demonstrated the presence of two high affinity binding sites: $K_D$, 0.10–0.20 nM and 2–4 nM, respectively. The high affinity complex constitutes about 30% of the total quantity of nuclear bound complexes. In addition two types of binding sites for $R_nE$ complexes were demonstrated by the resistance of $R_nE$ complexes to salt extraction by 0.4 M KCl. Immature rats were injected with estradiol and at various times after the injection the uteri were removed, homogenized and fractionated. The quantities of estrogen–receptor complexes in the cytosol ($R_c$), in 0.4 M KCl-extracts of the nuclear pellets ($R_n$-extractable) and in the nuclear pellet after KCl-extraction ($R_n$-resistant) were determined.

$R_c$ rapidly declined during the first 30 min and then increased slowly over the next two h. $R_n$-extractable increased rapidly during the first 20 min after injection and then dropped to control levels over the following 160 min. $R_n$-resistant increased slowly reaching a maximum after 60 min and then declining slowly during the subsequent 120 min.

When uterine nuclear fractions from rats treated with 2.5 μg of estradiol 1 h prior to sacrifice, were assayed for the quantity of receptor–steroid complexes, it was found that they contained about 5–6 times the amount found in the corresponding fraction from rats treated with 0.1 μg. About 80% of the receptor–hormone complexes found in the nucleus after injection of the high dose of estrogen was extractable by 0.4 M KCl, whereas the corresponding figure after the low dose treatment was approx. 50%.

These results show the presence of two classes of receptor–estradiol complexes with different affinities for the hormone. Both classes of complexes exist in the nucleus partly as KCl-extractable and partly as KCl-resistant. The shift from $R_n$-extractable to $R_n$-resistant form might reflect changes in the conformation of the $R_nE$ complexes upon association with specific acceptor sites.

## INTRODUCTION

Oestrogen sensitive cells contain cytoplasmic macromolecules called receptors [1, 2]. These macromolecules bind oestrogen to form receptor–estrogen complexes which undergo translocation to the nucleus where they may bind to specific acceptor sites [3–5]. The demonstration of acceptor sites in the nucleus has been the subject of considerable controversy [6, 7].

Most investigators have used *in vitro* systems in an attempt to show nuclear acceptor activity. The character of these binding sites in the nucleus is still a matter of some dispute. While some investigators have shown that nuclear binding of receptor–steroid complexes is a saturatable phenomenon [7–14] others claim that limited numbers of specific nuclear sites do not exist [6, 15, 16]. Some authors claim that the acceptor sites are located on DNA [17] while others find them mainly on non-histone proteins [12]. Much of this conflict probably stems from the inherent difficulty in the detection of a low number of specific binding sites in the presence of a large number of non-specific sites [17]. Thus, studies of the binding of the receptor–estrogen complexes to nuclei, chromatin and/or DNA under cell free conditions, are susceptible to the error introduced by the masking effect of non-specific binding [18].

Although the demonstration of nuclear acceptor binding is difficult *in vitro*, we have shown that a limited number of nuclear binding sites are involved in the production of maximal uterine growth [4, 5, 19]. We have also demonstrated that the long term retention of 1000–3000 receptor oestrogen complexes/cell is a requirement of uterine growth [19, 20]. Since uterine cells contain 15–20,000 receptor sites in the cytoplasm, it is apparent that only a fraction of these are required for maximal growth responses. These concepts and experiments are summarized in Fig. 1. We have suggested that the long term retention of the receptor oestrogen complex in the nucleus is due to the binding of these complexes to limited number of nuclear acceptor sites and that retention at these acceptor sites for greater than 4–6 h is a requirement for the production of true growth [5,21].

To test this hypothesis further we have used salt extraction of uterine nuclei to examine for differential extractability of the receptor oestrogen complex. The rationale for the use of this technique was based on the observation of several investigators that extraction of nuclei with 0.3–0.4 M KCl does not remove

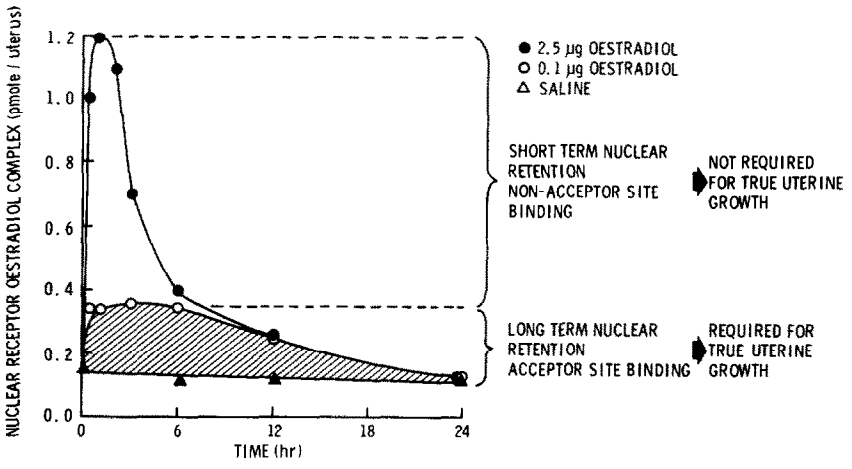


Fig. 1. Relationship between nuclear retention of the estrogen receptor and uterine growth. Immature rats were treated with either 0.1 or 2.5 μg of estradiol by sc injection and the accumulation and retention of the estrogen receptor by the uterine nuclear fraction was determined by the nuclear exchange assay [4, 31]. Uterine growth responses (DNA, RNA and protein content, wet and dry weight) were measured at 24–48 h after injection (data not shown, see refs. 4, 5, 19, 20) and were found to be maximally stimulated by 0.1 μg of estradiol.

all of the nuclear bound oestrogen [22–24], suggesting that some receptor hormone complexes are bound more tightly than others. We have also reexamined the affinity of the receptor for estradiol in the nuclear fraction. Thus, both binding of the hormone to its receptor site and the complex to nuclear sites have been analysed.

## EXPERIMENTAL PROCEDURE

For a detailed description of the methodology used see [25].

*Animals.* Immature female rats (21–23 days old) of the Sprague–Dawley strain were used. Injections of compounds were given as saline solutions subcutaneously. The animals were sacrificed by cervical dislocation.

*Cytoplasmic receptors.* Uterine cytosol was prepared by homogenization of the tissue in TE-buffer (0.01 M Tris–HCl-0.0015 M EDTA, pH 7.9) in an all glass homogenizer. The homogenate was centrifuged at 800 *g* for 20 min, the resulting supernatant was recentrifuged at 180,000 *g* for 60 min. The resulting high-speed supernatant was used for determination of cytoplasmic receptor according to the hydroxylapatite exchange assay [26].

*Nuclear receptors.* For the determination of nuclear receptor–hormone complexes, immature rats were injected with either a low dose (0.1 μg) or high dose (2.5 μg) of non-labelled estradiol. After the time indicated, the animals were killed and their uteri removed, cleaned and homogenized in cold TE-buffer. A nuclear pellet was prepared by centrifugation of the homogenate (30–50 mg/ml) at 800 *g* for 10 min. Although purified nuclei were not used in these experiments, they behave in a fashion similar to that of this crude nuclear preparation. The nuclear fraction was washed three times with cold TE buffer (0.01 M Tris, EDTA 0.0015 M, pH 7.9, 4°C). Salt extraction was performed by adding various concentrations of TK buffer (0.01 M Tris and 0.1–0.6 M KCl, pH 8.0, 4°C) to the nuclear pellet with the mass to vol. ratio maintained at 30–50 mg/ml. The nuclear pellets were resuspended in the TK buffer and allowed to stand on ice, with mixing every 5–10 min, for 30 min. Nuclear fractions were centrifuged at 800 *g* for 10 min and the KCl extract was decanted. TE buffer was added to the nuclear pellet (30–50 mg/ml) and the pellet was resuspended. If necessary, resuspension was accomplished by rehomogenization of the pellet with a glass–Teflon homogenizer. The quantities of receptor–steroid complexes in the KCl extract ($R_n$-extractable) and in the pellet after KCl-extraction ($R_n$-resistant) were determined by the hydroxylapatite-exchange assay and nuclear exchange assay, respectively, as described in detail elsewhere (25).

## RESULTS AND DISCUSSION

*Saturation curve.* Portions of a nuclear suspension obtained from uteri of immature rats treated with 2.5 μg of estradiol for 60 min, were incubated with increasing concentration of [$^3$H]-estradiol (0.1–16.0 nM or [$^3$H]-estradiol plus a 100-fold excess of DES). Figure 2A shows the binding curve obtained for the specifically bound hormone (total bound minus bound in the presence of a 100-fold excess of DES). Scatchard analysis [27] of these data (Fig. 2B) shows the presence of two classes of binding sites, with $K_D$'s of $3 \times 10^{-9}$ M and $3 \times 10^{-10}$ M, respectively, after correction according to Feldman [28]. The binding sites with the highest affinity constitute about 30% of the total amount of receptor sites.

*Characteristics of nuclear retention of receptor–estrogen complexes.* Uterine nuclear fractions from

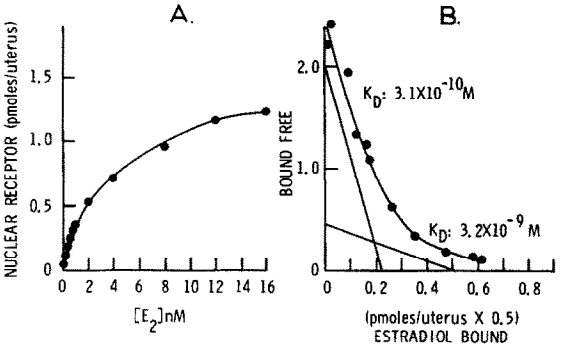


Fig. 2. Determination of the number of specific nuclear binding sites in the nuclear fraction of rat uteri from immature animals. (A) Rats were injected with 2.5 μg of estradiol. After 1 h the animals were killed and the uterine nuclear fraction was prepared as described under "Experimental procedures". This fraction was incubated for 30 min at 37°C with either [2,4,6,7-$^3$H]-estradiol alone or [2,4,6,7-$^3$H]-estradiol plus a 100-fold excess of DES. The specifically bound hormone was obtained by subtracting the amount of [$^3$H]-estradiol found with the nuclear fraction incubated with DES from the total nuclear binding in the absence of DES. (B) Scatchard analysis of the data presented in panel A.

rats treated with 2.5 μg of estradiol one hour before sacrifice contains a large number of receptor steroid complexes which are extractable with KCl concentrations less than 0.4 M (Fig. 3). The quantity of receptor which remains in the nuclear fraction after exposure to KCl concentrations of 0.4 M or higher is approximately 0.1 pmol/uteri or 1400 sites/cell. Thus, this small number of KCl resistant receptor sites could represent those receptor sites which exhibit

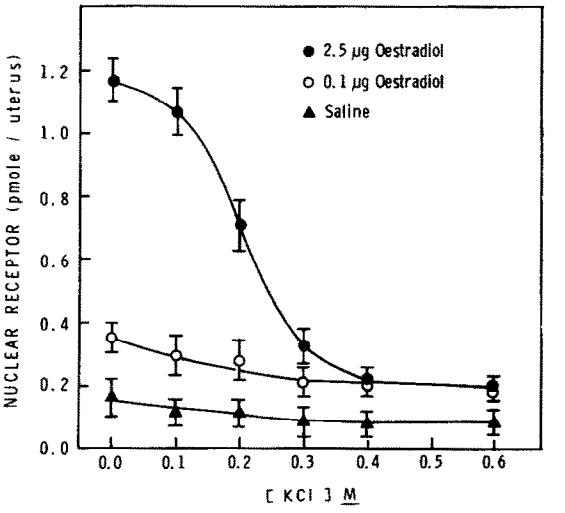


Fig. 3. Differential salt extraction of nuclear bound receptor-estradiol complexes. Immature rats were injected with estradiol as described in Fig. 1. Uterine nuclear fractions were prepared one hour after the injection and differential KCl extraction was performed as described in the text. After extraction, the quantity of receptor which remained in the nuclear fraction was measured as outlined in the text.

long term nuclear retention (Fig. 1). If this were true, the number of receptor–complexes bound in a KCl resistant manner in animals receiving the high dose of hormone should equal the number found in those receiving the low dose, since both high and low doses produce the same number of long term retained complexes (Fig. 1). As shown in Fig. 3, this appears to be the case. Likewise the number of KCl resistant receptor complexes should be equal 6 h after injection of either dose of oestrogen and should be approx. the same as the number at one hour after the low dose of oestrogen. This was also found to be the case. Therefore, we conclude that the number of receptor–steroid complexes which remain in the nucleus after 0.4 M KCl extractions, are correlated with the number of receptors which exhibit long term nuclear retention. The differential extraction of the receptor oestrogen complexes by KCl may reflect the binding of these complexes to different types of nuclear sites. The vast majority (80–90%) of these nuclear receptor binding sites are of low affinity and extractable with KCl ($R_nE$) while a minority (10–20%) are of high affinity and are non-extractable sites. In order to determine a possible relationship between $R_n$-extractable ($R_n^e$) and $R_n$-resistant ($R_n^r$) receptor–estrogen complexes, the disappearance of cytoplasmic receptors and the appearance of the two types of receptor–steroid complexes in the nucleus were measured as a function of time after exposure of immature rat uteri to estradiol *in vitro* or *in vivo*. The results of these experiments are shown in Fig. 4. Under *in vitro* conditions the concentration of $R_c$ declined rapidly during the first 60 min of incubation with a concomitant increase in the quantity of $R_n$-extractable complexes, reaching a maximum by 60 min. This was followed by a gradual increase in the quantity of $R_n$-resistant complexes which reached a peak by 150 min. This "transformation process" of $R_n$-extractable to $R_n$-resistant complexes was more rapid *in vivo* (Fig. 4B). $R_c$ decreased to a minimum by 30 min, $R_n$-extractable increased rapidly during the same time period and then dropped to control levels over the following 160 min, whereas $R_n$-resistant increased slowly reaching a maximum after 60 min and then declined slowly during the subsequent 120 min.

These data support a concept of a time dependent shift of nuclear estrogen receptor complexes from $R_n$-extractable to $R_n$-resistant binding sites, both *in vitro* and *in vivo*.

Observations made by other scientists have suggested that receptor hormone complexes manifest different characteristics once they have undergone nuclear binding. The rate of dissociation of the receptor oestrogen complex is much slower when bound to chromatin than when free [29, 30]. DeHertogh has observed a small pool of nuclear bound receptor *in vivo* which exhibits a relatively slow rate of estradiol exchange when compared to the majority of receptor [23]. We have also observed two rates of exchange of the receptor oestrogen complex when it is bound

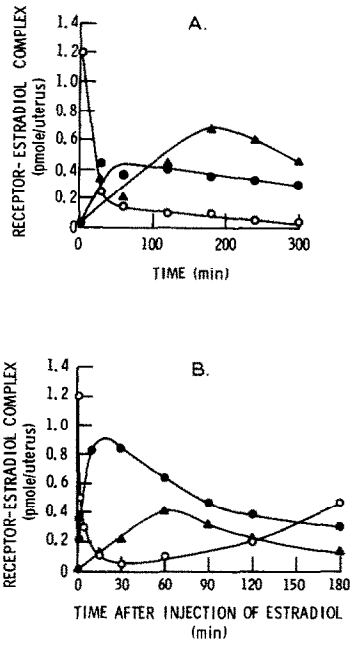


Fig. 4. Concentrations of uterine cytoplasmic and nuclear receptor–estrogen complexes as a function of time of *in vitro* incubation of intact uteri from immature rats (A) or time after injection of estradiol *in vivo* (B). (A) Uteri from 21-days old female rats were incubated at 37°C in Eagle's medium made 20 nM with respect to [2,4,6,7-$^3$H]-estradiol. After the time indicated in the Fig. the tissue was removed, washed with cold saline and homogenized in TE-buffer. Cytoplasmic-, Nuclear KCl-extractable and KCl-resistant receptor–estradiol complexes were determined as described in the text. (B) Immature female rats were treated with a subcutaneous injection of 2.5 μg estradiol, and killed at the time outlined in the graph. Their uteri were removed and the receptor concentration in the cytosol and nucleus determined as described in the text.
○——○: Cytoplasmic receptor–estradiol complex; ●——●: Nuclear KCl-extractable receptor–estradiol complex; ▲——▲: Nuclear KCl-resistant–estradiol complex.

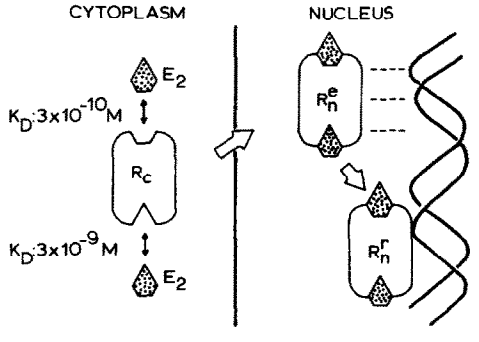


Fig. 5. Formation of receptor–estradiol complexes in the uterine cell, and their interaction with nuclear binding sites.

After interaction with the receptor binding sites the hormone is translocated as a receptor–steroid complex to the nucleus, where this complex first appears as a unit loosely bound to chromatin ($R_n^e$) and later much more strongly attached to chromatin ($R_n^r$).

to the nucleus (unpublished). The exact relationship of these various observations to these reported here are not clear at present; however, they may reflect conformational changes in receptor oestrogen complexes attendant to their association with specific nuclear acceptor sites. Such conformational alterations might result in associations of great affinity between receptor hormone complexes and acceptor sites, thereby accounting for their resistance to KCl extraction. Whether or not the high affinity receptor–estradiol complexes discussed above (Fig. 2) are more abundant among the $R_n$-resistant complexes or not has not yet been examined.

Preliminary data also suggests that the cytoplasmic form of the receptor contains two binding sites for estradiol (data not shown) that are similar to those described for the nuclear complex.

Thus, these results support a hypothesis (see Fig. 5) that the nuclear hormone receptors are one protein with two different binding sites for estradiol existing in two different states due to the localization of the receptor, the extractable form loosely bound to chromatin or in a hydrophilic environment, and the resistant form much more strongly chromatin attached or in a hydrophobic environment.

Possibly the extractable form serves as a storage form of active receptor–hormone complexes to guarantee a continuous binding to the chromatin located acceptor sites, as long as high levels of estrogen exist in the uterine cell. Since the number of the $R_n$-resistant nuclear sites is identical to that number of sites required for the production of maximal uterine growth, we propose that these binding sites in the nucleus may represent specific acceptor sites.

The presence of two different classes of binding sites for estradiol on the uterine receptor might be of significance in the nuclear processing of hormone during the so called "off-reaction". If a release of the hormone from its chromatin bound receptor is the first step in this reaction, a receptor interacting with its ligand with higher affinity would be expected to be retained for a longer period at its binding site, than would one interacting with a lower affinity.

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## DISCUSSION

*Junghlut.* It was nice to see that the old extractant still works, but I can hardly see that a single extraction with 0.3 M KCl is exhaustive. When you compare the extractability at various times after the administration of estradiol, is it not conceivable that the differences are due to changes in the tissue?

*Clark.* Yes, but this is conceivable. One thing that should be remembered is that we are measuring receptor site not estradiol. Therefore, we are measuring the receptor that is left in the nucleus not the estrogen and, thus, I believe KCl-resistant sites reflect tightly bound nuclear receptor. Whether these sites are of physiological significance or not will remain the subject of future studies.

*Bell.* The results of your *in vitro* studies remind me of some studies from Tomkin's group, where they showed that if nuclear sites were saturated with glucocorticoids in the intact cell, they could still accomplish cell-free binding of the steroid–receptor complex to nuclei, implying that the binding to isolated nuclei may not be to the same sites. You also stated that the early binding, the salt-extractable binding, is probably non-specific, I think?

*Clark.* The extractable complexes probably are non-specific. We think the non-extractable complexes are those of importance and reflect specific binding in the nucleus.

*Bell.* It would seem to me that the time course of RNA polymerase stimulation coincides much better with the salt-extractable binding.

*Clark.* That's not true, as we have examined RNA polymerase activity and it correlates very well with the non-extractable complexes.

*Lippman.* You were saying that if you just prepare cytoplasmic extracts and do careful binding analysis that you now show there were 2 sites whether on different protein or not of different affinity but you get a split Scatchard, if that's so many people have done that sort of thing and presumably I think it's a little glib to suggest simply that they have not used a high enough concentration of estrogen to see that. Is that something of a general phenomen? Have you looked for this in other tissues? How do you put this together?

*Clark.* We have asked ourselves that very question and when we examined the literature, we observed that various labs can be divided into two groups (A and B). A-groups use very low concentrations of estradiol to do saturation analysis and B-groups (such as ours) always use higher concentrations. This results in each group ignoring values at high or low concentrations respectively,—thus, one or the other of the components has been overlooked.

*Pasqualini.* The data of the presence of nuclear resistant sites is an intriguing problem. In this relation, in our laboratory many years ago (*C.r. hebd. Séanc. Acad. Sci.* (Paris) **273** (1971) 1061–1063; *J. steroid Biochem.* **3** (1972) 543 and *C.r. hebd. Séanc. Acad. Sci.* (Paris) **276** (1973) 3359) we found *in vivo* and *in vitro* using either [$^3$H]-aldosterone or [$^3$H]-estradiol that in the fetal kidney of guinea pig only 20–30% of the total nuclear radioactivity was extracted by 0.1 M Tris and 0.3 M NaCl solutions. On the other hand, most of the radioactivity (50–60%) was successively extracted by solutions of 1 M NaCl and the remaining by 3 M NaCl; 0.2 NHCl; 0.2 N NaOH, and ethanol.