Estrogen Receptors in the Rat Uterus

STUDIES ON THE INTERACTION OF CYTOSOL AND NUCLEAR BINDING SITES*

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SUMMARY

Studies on the interrelationship of binding sites for estrogens found in cytosol and nuclear fractions of the rat uterus show that 17β-estradiol is first associated with a protein (9 S) present in the cytosol. The estrogen subsequently moves into the nucleus where it is bound to a protein (5 S) which can be extracted from the chromatin. Simultaneously with the movement of the estrogen into the nucleus, the binding protein of the cytosol disappears or loses its ability to bind estrogen.

These and other experiments are discussed in terms of a hypothetical model in which estrogen binds to a cytosol receptor protein, bringing about a change in its conformation and its subsequent movement into the nucleus.

Recent studies (6, 7) on estrogen binding in the soluble fraction of the rat uterus indicated that the binding molecule is a large protein having an approximate sedimentation coefficient of 9 S, and that the binding is saturated at relatively low concentrations when estrogen is administered in vitro and is specific for estrogenic molecules and for tissues of the reproductive tract. The foregoing are consistent with characteristics of an estrogen receptor.

Since these studies showed that estrogen was retained in significant amounts in both the cytosol and nuclear-myofibrillar fractions the investigations reported here were designed to determine whether the binding characteristics in these two fractions are the same and whether the binding molecules are related.

Methods

Isolation of Pure Nuclei from Immature Rat Uteri—Uterine nuclei were isolated by the modification of a procedure for the preparation of nuclei from rat diaphragm.1 These nuclei were microscopically normal and were capable of synthesizing RNA.2 Immediately after removal, the uteri were homogenized with a Virtis model 23 for 45 sec at high speed in a medium of sucrose (0.25 M), calcium chloride (0.003 M), and Tween 603 (0.5% w/v). The homogenate was stirred for 15 min and filtered through a layer of flannel. The filtrate was then stirred for 5 min and centrifuged at 800 X g for 15 min at 4°. The pellet obtained consisted of nuclei which appeared to be relatively free of myofibrils and cell debris when examined under a phase contrast microscope. Unless otherwise stated, before use the pellets were washed twice with 0.01 M Tris-Cl buffer, pH 7.4, at 0°, each time by centrifugation at 800 X g for 15 min at 4°. The pellet obtained consisted of nuclei which appeared to be relatively free of myofibrils and cell debris when examined under a phase contrast microscope.

Preparation of Chromatin from Isolated Nuclei—The procedure for isolating chromatin was an adaptation of that used by Marushige and Bonner (8) for isolating liver chromatin. The nuclear pellet, suspended in 0.05 M Tris-Cl, pH 7.4 (0.2 ml per uterus), was centrifuged at 10,000 X g for 15 min. This step was repeated and the resulting sediment was suspended in 5 to 7 ml of 0.05 M Tris-Cl, pH 7.4, by homogenizing in a Teflon ho-

1 J. Kostyo, personal communication.
2 J. Barry and J. Gorski, unpublished data.
3 Polyoxyethylene sorbitan monostearate (Sigma).
TABLE I  
Incorporation of \(^{3}H\)-estradiol into isolated nuclei and chromatin of immature rat uterus

Fifteen rats (21 days old) were injected with 0.05 \(\mu\)g of estradiol-\(^{17}\beta\)-\(^{3}H\) and killed at the times indicated. Uteri were removed and processed as described under “Methods.”

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total recovered activity at 1 hr</th>
<th>Total recovered activity at 3 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>Homogenate</td>
<td>76,230</td>
<td>73,260</td>
</tr>
<tr>
<td>Washed nuclei</td>
<td>12,000</td>
<td>12,141</td>
</tr>
<tr>
<td>10,000 (\times g) supernatant</td>
<td>212</td>
<td>189</td>
</tr>
<tr>
<td>Sucrose supernatant</td>
<td>2,000</td>
<td>600</td>
</tr>
<tr>
<td>Chromatin pellet</td>
<td>8,385</td>
<td>7,884</td>
</tr>
</tbody>
</table>

TABLE II  
Concentration of \(^{3}H\)-estradiol bound to nuclei and chromatin of immature rat uterus

Fifteen rats (21 days old) were injected with 0.05 \(\mu\)g of estradiol-\(^{17}\beta\)-\(^{3}H\) for 1 hr. Uteri were removed and processed as described under “Methods.”

<table>
<thead>
<tr>
<th>Cell preparation</th>
<th>Bound estradiol</th>
<th>DNA</th>
<th>Protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/nterus</td>
<td>(\mu)g/nterus</td>
<td>(\mu)g/nterus</td>
<td>cpm/(\mu)g DNA</td>
</tr>
<tr>
<td>Homogenate</td>
<td>5082</td>
<td>219</td>
<td>2448</td>
<td>23.2</td>
</tr>
<tr>
<td>Nuclei</td>
<td>800</td>
<td>72</td>
<td>225</td>
<td>11.1</td>
</tr>
<tr>
<td>Crude chromatin</td>
<td>786</td>
<td>66</td>
<td>224</td>
<td>11.9</td>
</tr>
<tr>
<td>(10,000 (\times g) pellet)</td>
<td>559</td>
<td>47</td>
<td>100</td>
<td>11.8</td>
</tr>
</tbody>
</table>

The homogenizer was layered on 20 ml of 1.7 M sucrose in Tris-Cl buffer (0.01 M, pH 7.4, at 0°). The upper two-thirds or each tube was then gently mixed. The tubes were centrifuged at 22,000 rpm for 3 hours in the Spinco SW 25 rotor to obtain the chromatin pellet. The recovery of nuclear DNA in the chromatin ranged from 70 to 90%. The chemical composition, of the chromatin (mass ratio: DNA 1.00, RNA 0.22, protein 1.72; histone 1.15) is similar to that reported for chromatin isolated from uteri of ovariectomized rats (9). The absorption spectrum of the chromatin preparation indicates a ratio of absorbance at 280 nm to that at 260 nm of 0.65, also implying a high nucleic acid content.

In some cases the nuclear or chromatin pellet was extracted with 0.3 M KCl in 0.2 M phosphate buffer, pH 7.4 at 0°. The resulting viscous solution, centrifuged at 220,000 \(\times g\) for 30 min, yielded a salt extract similar to that described by DeSombre et al. (10).

Preparation of Cytosol and Particulate Fractions—Whole uteri were incubated in 2 ml of Eagle’s medium (11) at 6° or 37° in an atmosphere of 95% \(O_2\)-5% \(CO_2\). Homogenates of washed uteri were prepared in 0.01 M Tris-Cl buffer, pH 7.4, and centrifuged at 120,000 \(\times g\) for 30 min to yield cytosol and particulate fractions.

Sucrose Density Gradient Analysis—The samples to be analyzed (usually 0.2 ml) were layered on 10 to 30% sucrose gradients in 0.01 M Tris-Cl buffer, pH 7.4 (in the case of nuclear and chromatin preparations), on 5 to 20% sucrose gradients in 0.01 M Tris-Cl buffer, pH 7.4 (in the case of cytosol). They were centrifuged for indicated time intervals at 220,000 \(\times g\) at 3° in an International model B-60 with an SB-405 rotor. The fractionation and analysis of these samples were done according to methods described elsewhere (6). Approximate sedimentation coefficients were determined by the method of Martin and Ames (12) with bovine liver catalase as the standard. Aliquots of various samples were treated with 500 \(\mu\)g of trypsin or Pronase and 1000 \(\mu\)g of DNase or RNase at 0° for 30 min to determine the nature of the binding sites.

Chemical Analysis—DNA and RNA were fractionated by the Schmidt-Thannhauser Procedure (13). DNA was determined by the diphenylhydrazine method (14) with sperm DNA as a standard. RNA was determined by the procedure described by Ceriotti (15) with yeast RNA as a standard.

Histones were extracted from chromatin with 0.2 n HCl at 2-3° for 30 min and precipitated with 20% trichloracetic acid. Nonhistone proteins were isolated from the 0.2 n HCl-insoluble fraction after removal of nucleic acids by heating at 95° for 15 min in 10% trichloracetic acid.

Protein was determined by the method of Lowry et al. (16) with bovine serum albumin as a standard.

17\(\beta\)-Estradiol-6,7-\(^{3}H\) (specific activity 42 to 48 Ci per mmole) was obtained from New England Nuclear and was routinely checked for radiopurity by paper chromatography.

RESULTS AND DISCUSSION

Intranuclear Localization of Bound Estrogen—Table I shows the incorporation of \(^{3}H\)-estradiol-\(^{17}\beta\) administered in vivo into the various fractions which results during the process of extracting pure nuclei and chromatin from the immature rat uterus. Table II expresses the binding of estrogen to different cell fractions on the basis of their DNA and protein content. Approximately 15% of the total estradiol and 30% of the total DNA from the homogenate were recovered in the nuclei. This disparity in the recoveries of DNA and estradiol is explained by the finding that only about 50% of the total bound estrogen is associated with the nuclear-myofibrillar fraction (5). Both of the recoveries were greater when homogenization times were longer, but this invariably resulted in more cytoplasmic contamination of the nuclei. Since it was important to obtain pure nuclei before the isolation of chromatin, shorter homogenization periods were used throughout this study. The recoveries of DNA and estradiol from nuclei into the chromatin were 71% and 70%, respectively. Whether the animals were injected with estradiol for 1 or 3 hours prior to killing, the recoveries of radioactivity were similar. Since most of the excess estrogen is known to be lost from the uterus by 3 hours (2, 5), the estrogen associated with chromatin and nuclei appears to be due to specific binding rather than nonspecific binding of the excess estrogen. In subsequent experiments varying doses of diethylstilbestrol injected 30 min prior to radioactive estrogen administration reduced the uptake of \(^{3}H\)-estradiol in nuclei and chromatin proportionate to the levels of diethylstilbestrol administered. This confirmed the specificity of the binding in the nuclei and chromatin.

To determine the nature of the binding site in the chromatin and nuclei, various enzymatic treatments were carried out as described under “Methods.” About 60 to 70% of the bound estrogen was released from both chromatin and nuclei with proteolytic enzymes, about 10 to 20% by DNase, and no significant amount was released as a result of RNase treatment.
Subsequent analyses on sucrose gradients of material released by DNase or proteolytic enzymes indicated that the released estrogen was free rather than bound. Although 75 to 80% of the bound estrogen in the chromatin was released by treating with the 0.2 M HCl used to extract histones, sucrose gradient analysis revealed that the acid extract contained only free estrogen. Low pH is known to affect the binding of estrogen to the receptor in the nuclear-myofibrillar fraction; hence these results do not offer any suggestion as to the role of histones in the binding phenomenon (5).

Recently it has been shown that bound estrogen from the nuclear fraction of the rat uterus or calf endometrium can be extracted by a single freezing and thawing in the presence of 0.3 M KCl (10). This extract contained an estrogen-receptor complex which has a sedimentation value of 5 S when analyzed on sucrose density gradients. We were able to confirm these findings with extracts of pure nuclei. Chromatin preparations treated with DNase before extracting with 0.3 M KCl also yielded extracts containing bound estrogen sedimenting at approximately 5 S (Fig. 1). When salt extracts from nuclei were treated with Pronase and trypsin, as described under "Methods," estrogen binding was destroyed, whereas nucleases had no apparent effect.

Incubation of Estradiol with Isolated Whole Uteri—When whole uteri were incubated at 37° in Eagle's medium containing known amounts of radioactive estrogen, binding was observed in the nuclei and subsequent analysis of salt extracts revealed a 5 S component. No such binding was observed when the incubation was done at 0° (Fig. 2).

Isolated nuclei, chromatin, and 0.3 M KCl extracts of these preparations failed to bind estrogen in vitro at 0°. At 37°, significant amounts of estradiol were retained by nuclei under cell-free conditions; however, analysis of the 0.3 M KCl extracts of these preparations on sucrose density gradients indicated that the retained estradiol was not bound. Moreover, at 37° in cell-free systems, significant amounts of testosterone and progesterone were also retained by the nuclei. Thus the uptake of estradiol at 37° by the nuclei under cell-free conditions seems to be a nonspecific phenomenon. Earlier works (3) have also shown the failure of the nuclear-myofibrillar fraction in cell-free systems to bind estradiol.

Studies in Vitro on Distribution of 1°H-Estradiol-17β between Cytosol and Particulate Fraction—Fig. 3 shows the distribution of 1°H-estradiol-17β between particulate and cytoplasmic fractions when whole uteri are incubated with estradiol at 0° or 37° for various lengths of time. It is apparent that after 30-min incubation at 37°, the distribution of 1°H-estradiol does not differ substantially from that found after administration in vivo of the hormone. At 0°, however, most of the estrogen retained by the uterine was present in the cytosol.

Fig. 4 shows the results of experiments in which uteri were incubated first with 1°H-estradiol at 0° for 1 min and then trans-
Fig. 3. The distribution of radioactive estradiol between cytosol (120,000 × g × 30 min) and particulate fraction after whole tissue incubation in vitro. In each group two uteri from immature rats were incubated in 2 ml of Eagle's medium containing 0.005 μg of estradiol-17β-3H at 0° or 37° for various times as indicated.

Fig. 4. The release of radioactive estradiol from cytosol into particulate fraction upon incubation at 37°. In each group two uteri were incubated at 0° for 1 min in 2 ml of Eagle's medium containing 0.02 μg of 3H-estradiol-17β. The uteri were then transferred to fresh medium without any estradiol and incubated at 37° for various times as indicated.

Fig. 5. Sucrose density gradient patterns of uterine cytosol. In each group four uteri were incubated at 0° for 1 min in 2 ml of Eagle's medium containing 0.05 μg of 3H-estradiol-17β. The uteri were then transferred to fresh medium without any estradiol and incubated at 37° for various times as indicated.

Refer to an estrogen-free medium and incubated for various lengths of time at 37°. Under these conditions, there is a loss of 3H-estradiol from the cytosol and a proportionate gain of radioactivity in the particulate fraction. The total counts taken up by the uteri in each time group were approximately the same and there was no significant loss of radioactivity in the transfer medium as a result of incubation at 37°.

In some cases in which the uteri were first treated as described in Fig. 4 and then incubated in a third medium, free of estrogen, at 0° for various times, no estradiol was released into the medium. The purpose of this experiment was to determine whether the radioactivity from the particulate fraction could migrate back to the cytosol. Our results indicated that this did not occur. Thus it appears that the estradiol moving from the cytosol to the particulate fraction is not simply the result of diffusion influenced by temperature.

Fig. 5 shows the sucrose density gradient analysis of cytosol preparations from uteri incubated under conditions described in Fig. 4. At 0°, the 3H-estradiol retained by the cytosol was associated mainly with the 9 S region of the gradient, and subsequent incubations at 37° reflected a loss of radioactivity from that region. It is possible that, in our system, the estradiol-receptor complex dissociated with unbound estradiol moving into the particulate fraction, leaving behind the free receptor. In subsequent experiments, the cytosol prepared from uteri under conditions identical with those described in Fig. 5 were further treated with 3H-estradiol under cell-free conditions. The receptor in the cytosol is known to bind estrogen directly added under cell-free conditions. The receptor in the cytosol is known to bind estrogen directly added under cell-free conditions (7).

In order to determine whether it was necessary for the receptor to bind with estrogen before it moved into the nucleus, uteri were incubated at 37° in a medium without any estrogen and 3H-estradiol was added directly to the cytosol. Density gradient analysis of the cytosol from these experiments show that binding of 3H-estradiol still occurs in the 9 S region (Fig. 7). Apparently, the 9 S receptor disappears from the cytosol with 37° incubation only in the presence of estradiol. This implies that there is a selective transfer of estradiol-receptor complex from the cytosol at 37°.

Similar results were obtained when the above experiments were repeated with 3H-estriol instead of 3H-estradiol. This is significant since 3H-estriol, a more polar steroid, does not bind to the 4 to 6 S material of the cytosol. It is interesting to note that in the 4 to 6 S region there was no binding of estradiol as occurred in the cytosol prepared from unincubated uteri. The binding of...
FIG. 6. Sucrose density gradient patterns of uterine cytosol after whole tissue incubations followed by cell-free incubation. Treatment of the uteri was as follows: G-1, four uteri were incubated at 37° for 1 hour in 2 ml of Eagle's medium; G-2, four uteri were incubated at 0° for 1 min in 2 ml of Eagle's medium containing 0.05 μg of 3H-estradiol-17β; G-3, four uteri were incubated at 0° for 1 min in 2 ml of Eagle's medium containing 0.05 μg of 3H-estradiol-17β and then transferred to fresh medium without estrogen and incubated for 1 hour at 37°. In each case 0.005 μg of 3H-estradiol-17β was added directly to 0.4 ml of cytosol. Samples, 0.2 ml, were layered on 5 to 20% sucrose gradients and centrifuged at 48,000 rpm for 14 hours at 4°C. Counting efficiency was 19 to 20%. Data are adjusted to represent one uterus.

Fig. 7. Sucrose density gradient patterns of uterine cytosol. Treatment of the uteri was as follows: G-1, uteri were not incubated; G-2, uteri were incubated for 1 hour at 37° in 2.0 ml of Eagle's medium without estrogen. In both cases cytosol was prepared as described under "Methods," 3H-Estradiol-17β, 0.005 μg, was directly added to 0.4 ml of cytosol. Samples, 0.2 ml, were layered on 5 to 20% sucrose gradients and centrifuged for 15 hours at 55,000 rpm at 4°C. Counting efficiency was 20%.

estrogen to 4 to 6 S region is believed to be caused by serum proteins (7). It would seem that following incubation at 37° although the 9 S receptor remains in the cytosol, the material in the 4 to 6 S region which binds estradiol disappear, probably into the incubation medium.

Both uptake and transfer of estradiol from cytoplasm was unaffected by addition of cycloheximide (10 μg per ml) or actinomycin D (20 μg per ml) to incubation and transfer media. This indicates that both the initial uptake in the cytosol and subsequent transfer of estrogen into the particulate fraction precede other metabolic responses to the hormone. Transfer of the hormone also appeared to be independent of energy availability since the translocation was observed even when the incubations at 37° were done (a) under an atmosphere of nitrogen or (b) in the presence of either 1 to 2 mM cyanide or 0.2 mM 2,4-dinitrophenol.

When the uteri were first incubated at 0° with estradiol and subsequently transferred to a medium free of estradiol but containing 10−3 M N-ethylmaleimide, 10−3 M iodoacetamide, and 10−3 M p-chloromercuribenzoate, uptake of the hormone by the particulate fraction was completely abolished. Transfer medium containing 10−4 M ouabain did not affect transfer of estradiol from cytosol to particulate fraction.

In studying the rate of transfer of estrogen from cytosol to the particulate fraction, the Michaelis and Menten theory (17) of enzyme kinetics was used. This involves the following assumptions.

1. The receptor in the cytosol first forms a complex with estradiol described according to the equation:

\[
[E] + [S] \rightleftharpoons (ES)
\]

(Receptor) + (estradiol) ⇌ (complex)

and ES subsequently moves into the particulate fraction.

2. The equilibrium for the binding of estradiol to the receptor in the cytosol tends toward formation of the complex and, once ES is transferred to the particulate fraction, the release of the complex back into the cytosol is not significant enough to affect the equilibrium of the over all reaction.

3. The number of binding sites in the uterus is unchanged during this uptake and transfer of estradiol.

We have presented evidence in this paper that estradiol initially interacts with only the cytosol receptor to any significant extent. It has been shown that the binding of estradiol to the
Finally, since the extent of uptake as well as the transfer of particulate fraction, it does not move back into the cytosol. We have also found that once estradiol has been moved into the amount of estrogen (8) present inside the cells and bound to concentration in the external medium. Therefore estimates of $K_v$, the receptor-estradiol complex was estimated to be $8.4 \times 10^{-13}$ moles per uterus. This value is not dependent on knowing the exact internal concentration of $S$ and should be accurate within experimental error. In the cytosol, the receptor-estradiol complex has a dissociation constant of $7 \times 10^{-9}$ M; the receptor-estradiol complex has a dissociation constant of $2 \times 10^{-9}$ M, and the concentration of estradiol-binding sites is estimated to be $9.0 \times 10^{-9}$ moles per uterus (7).

**GENERAL DISCUSSION**

Localization of estrogen in the purified nuclei after binding in vivo indicates that the estrogen observed in the nuclear-myofibrillar fraction (5) was indeed due to estrogen binding in the nuclei and, more specifically, in the chromatin. King, Gordon, and Martin have suggested that, in the case of rat anterior pituitary glands and in dimethylbenzanthracene-induced mammary tumors, estradiol might affect cellular function by its association with the chromatin (10). The component in the receptor responsible for the binding apparently is a protein and therefore is similar to the receptor in the cytosol. Thus this study suggests that estrogen action does not involve direct linking of estrogen to nucleic acids (20) or nucleotides (21).

The most notable observation of this investigation is the sequential movement of estrogen first into the cytoplasm (where it is bound to a large, approximately 9 S protein) and then into the nucleus. Several lines of evidence support a model in which the estrogen-9 S binding protein moves as a complex into the nucleus. The 9 S binding disappears from the cytosol simultaneously with the movement of estrogen into the nucleus. Moreover, the concentration of binding sites in the nucleus from these experiments in vitro is estimated to be about the same as Toft, Shyamala, and Gorski (7) reported for the cytosol. The dissociation constant for the nucleus determined here from the $K_w$ can only be a rough approximation but is similar to that of the cytoplasm (7). The affinity for the 9 S receptor is high enough that transfer of estrogen to a different nuclear receptor would probably necessitate a still higher affinity receptor. Unfortunately, no direct evidence is available to show that the 9 S receptor enters the nucleus. When both cytosol and nuclei are treated together with estradiol under cell-free conditions at $0^\circ$ or $37^\circ$, the nuclei still do not bind estrogen in a specific manner. Recently Brecher et al. (22) reported that binding to the nuclear does occur in homogenates at $25^\circ$. An explanation for the great difference in sedimentation characteristics of the 9 S cytosol receptor and the 5 S estrogen-binding agent extracted with $0.3 \text{ M} KCl$ from the nucleus is obviously needed. Studies presently underway will attempt to characterize further these two binding forms and the influence of high salt concentrations on their physical properties.

The movement of estrogen into the nucleus in association with the 9 S receptor has important implications in the primary mechanism of action of this hormone. One may speculate then that the primary function of the estrogens is to initiate a conformational change in the cytosol receptor protein, permitting its movement into the nucleus. Since publication of our preliminary report and while this manuscript was in preparation, Jensen et al. (23) have reported data which has led them to similar conclusions in regard to the estrogen receptors in the rat uterus.

**REFERENCES**

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G. Shyamala and Jack Gorski

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