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Estrogen Receptors in the Rat Uterus. Retention of Hormone–Receptor Complexes†

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ABSTRACT: The retention pattern and biochemical characteristics of estrogen receptors in the nuclei of uterine cells were studied as a function of time after the in vivo injection of estradiol (E_2) to immature female rats. One hour after the injection of 0.1 μg of tritiated E_2, approximately 0.20 pmol per uterus of receptor bound hormone is retained in uterine nuclei. This dose of E_2 produces a maximal uterotrophic response. Six hours after E_2 administration, uterine nuclei retain 0.04–0.08 pmol of hormone per uterus. Hormone receptor complexes extracted from uterine nuclei 1, 3, and 6 h after in vivo injection of hormone have similar structural and binding characteristics. Receptors extracted at all three times sediment at 5S in high salt gradients and have a dissociation binding constant of approximately 3 nM for E_2. The wash-out curves of receptors as a function of salt concentration are identical for uterine nuclei from animals treated for 1 or 6 h with estradiol, suggesting that the nature of the nuclear binding of receptors is not altered during this time interval. Experiments utilizing the injection of unlabeled estradiol, followed by an in vitro exchange procedure with tritiated estradiol, indicated that the total nuclear estrogen receptor sites, i.e., filled and vacant, decreased similarly.

The initial event in the action of a steroid hormone with a target tissue is thought to be the formation of a complex between the hormone and a specific cytoplasmic receptor protein. Following hormone-receptor interaction in the cytoplasm, the complex appears to migrate to the nucleus of the target cell, where it is bound to nuclear components, or nuclear acceptor sites. This nuclear localization of the receptor–hormone complex is thought to stimulate transcription, and thus initiate a sequence of biochemical events which ultimately culminate in the overall physiological changes produced by the hormone (Gorski, 1973; Williams, 1974; Jensen and DeSombre, 1973). A number of different steroid hormones studied to date appear to exert their effects via this general type of "cascade" mechanism (Jensen and DeSombre, 1972; O'Malley et al., 1971).

One system which has been extensively studied is the stimulation of the immature rat uterus by estrogens. Numerous studies with this system have focused on the characterization of estrogen receptors (Puca et al., 1972; Stancel et al., 1973a,b; Jensen et al., 1971), the nuclear localization of estrogen receptors (Shymala and Gorski, 1969; Jensen et al., 1968; Puca et al., 1974), and the effects of estrogen treatment on uterine RNA synthesis (O'Malley and Means, 1974; Hamilton, 1971; Glasser et al., 1972; Gorski, 1964). In this communication we present the results of studies on the nuclear retention patterns of estrogen receptors in uterine nuclei and characteristics of estrogen receptors present in uterine nuclei at various times after hormone administration.

Our interest in this area was stimulated by a provocative series of reports which raised the interesting possibility that different physiological responses of the uterus to estrogens might result from the nuclear interactions of estrogen receptors at different times after hormone administration (Anderson et al., 1972a, 1973, 1975). It therefore seemed important to us to attempt to define the nuclear retention patterns of uterine estrogen receptors, and to determine if receptors were modified in uterine nuclei.

Materials and Methods

Animals. Immature female rats (20–21 days old) were obtained from Texas Inbred Mice Co., Houston, Texas. Tritiated estradiol (48 Ci/mmol, New England Nuclear) or unlabeled estradiol (Schwarz/Mann) were given intraperitoneally in a volume of 0.5 ml of saline containing 5% ethanol. At the indicated times the animals were sacrificed by decapitation and the uterus removed and stripped of adhering fat and mesentery.

Isolation of Nuclei. Uteri were homogenized in 0.01 M Tris, pH 7.4, containing 1.5 mM EDTA[1] (TE buffer) using a ground-glass homogenizer. Crude nuclear-myofibrillar pellets were then obtained by centrifugation at 800*g* for 10 min. For the studies of total nuclear content of radioactive

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[1] Abbreviations used are: E_2, estradiol; EDTA, ethylenediaminetetraacetic acid; TE buffer, Tris-EDTA.

estradiol (E_2), each uterus was homogenized in 2.0 ml of TE buffer, and the nuclear pellets were washed three times with 3.0 ml of TE buffer. The pellets were then extracted with 3.0 ml of EtOH and the extracts counted for tritium content.

The nuclear exchange assay was performed as described by Anderson et al., (1972b). For each determination two uteri were homogenized in 2.0 ml of TE buffer, and the nuclear pellet obtained after centrifugation was washed three times with 1.5 ml of TE buffer. The washed pellet was suspended in 2.0 ml of TE buffer, and duplicate aliquots (0.25 ml) were assayed for estrogen receptor content by the addition of 100 μl of a stock tritiated estradiol solution (45.5 nM) in TE buffer. This yielded a final tritiated estradiol concentration of 13 nM. The samples were then incubated at 37 °C for 30 min and nuclei collected by centrifugation at 0 °C. After three washes with cold TE buffer, the pellets were extracted with 2.0 ml of EtOH, and the extract was counted for tritium content. Nonspecific estradiol binding was monitored by incubating aliquots of the nuclear suspensions with 13 nM tritiated estradiol plus a 100-fold excess of unlabeled diethylstilbestrol. The data presented in the Results section have all been corrected for this nonspecific binding.

Sucrose Gradients. Groups of six uteri were homogenized in 5.0 ml of TE buffer. A crude nuclear-myofibrillar pellet was then prepared by centrifugation at 800*g* for 10 min and washed three times with 3.0 ml of TE buffer. The volume of the final pellet was estimated, and an equal volume of TE buffer containing 0.8 M KCl was added. After a 30-min incubation at 0 °C, the samples were centrifuged at 100 000*g* for 30 min. Following centrifugation 200-μl aliquots of the supernatant were layered on linear 5–20% sucrose gradients.

The gradients were prepared in TE buffer containing 0.4 M KCl as previously described (Stancel et al., 1973a). The samples were centrifuged at 0–4 °C for 16 h at 50 000 rpm in a Beckman SW 50.1 rotor. ^{14}C-labeled ovalbumin was used as a marker and run on a separate gradient in the same rotor.

Salt Wash-Out Curves. Groups of four uteri were homogenized in 5.0 ml of TE buffer and the nuclear pellets prepared and washed as above. The pellets were then suspended in 5.5 ml of TE buffer. One-milliliter aliquots of the suspended nuclei were pelleted by centrifugation, and the pellets obtained were then suspended in 1.0 ml of TE buffer containing the indicated concentrations of KCl, and the suspensions were incubated at 0 °C for 20 min. The nuclei were then centrifuged at 800*g* for 10 min and washed three times with 3.0 ml of TE buffer. The washed pellets were then extracted with 3.0 ml of EtOH and extracts counted for tritium content.

Results

The studies performed in this work were done following an injection of 0.1 μg of estradiol to immature female rats. This dose was chosen since it produces a maximum or near-maximum increase in uterine growth measured 24 h after hormone treatment. Uterine growth was measured in the following manner. Animals were injected with various doses of estradiol and sacrificed 24 h later. Uterine weights and the in vitro incorporation of tritiated thymidine into DNA of surviving uteri were then measured. In both cases a maximum response was obtained between 0.05 and 0.2 μg of estradiol.

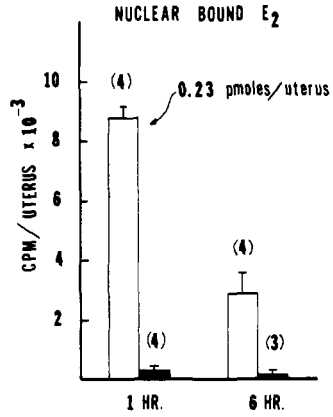

FIGURE 1: Nuclear bound estradiol 1 and 6 h after hormone treatment. Animals were injected with 0.1 μg of ^{3}H-E_2 (open bars) or 0.1 μg of ^{3}H-E_2 plus 10 μg E_2 (solid bars). Radioactivity in uterine nuclei was determined 1 and 6 h after hormone injections. Values represent means and the bars indicate SEM. The number of animals in each determination is given in parentheses.

After the administration of 0.1 μg of tritiated estradiol, the retention pattern of labeled hormone was observed in uterine nuclei; this is illustrated in Figure 1. One hour after administration approximately 0.2 pmol of hormone per uterus was localized in the uterine nuclei, but 6 h after treatment uterine nuclei contain only 30% of this amount (Figure 1). The open bars in Figure 1 illustrate the radioactivity present in the nuclear fraction per uterus following the administration of 0.1 μg of tritiated estradiol alone. The closed bars represent the radioactivity per uterus after administration of 0.1 μg of tritiated estradiol plus a 100-fold excess of unlabeled estradiol, and indicate that only negligible nonspecifically bound hormone is present in uterine nuclei. These studies indicate that the amount of nuclear receptor–hormone *complex* decreased between 1 and 6 h after hormone administration. That is, we measured only those receptors labeled with bound tritiated estradiol, rather than the total amount of receptors present, i.e., receptors with filled or vacant hormone binding sites. We therefore studied the retention of total receptor sites using the nuclear exchange assay (Anderson et al., 1972b), which should measure both filled and vacant receptor sites.

In these studies, animals were injected with unlabeled estradiol and sacrificed at different times after treatment, and nuclei were isolated. The isolated nuclei were then incubated at 37 °C with tritiated estradiol which exchanges with any bound estradiol. The tritiated estradiol would also be expected to bind to any vacant hormone binding sites. The results of such an experiment are illustrated in Figure 2.

Animals were injected with 0.1 μg of unlabeled estradiol or saline alone and the quantity of receptors in the nuclear fraction at 1 and 6 h after treatment was determined by the nuclear exchange assay (Anderson et al., 1972b). These values are given in panel A, Figure 2. Uterine nuclei from control animals which did not receive estrogen treatment appear to contain some estrogen (sample C). This has been repeatedly observed in studies using the nuclear exchange assay (Anderson et al., 1972a, 1973, 1975) and presumably reflects low levels of circulating estrogens. The values for the 1- and 6-h time points were therefore corrected for this control level and are illustrated in Figure 2, panel B. The results shown in this figure have been corrected for nonspecific binding as described in the Materials and Methods section.

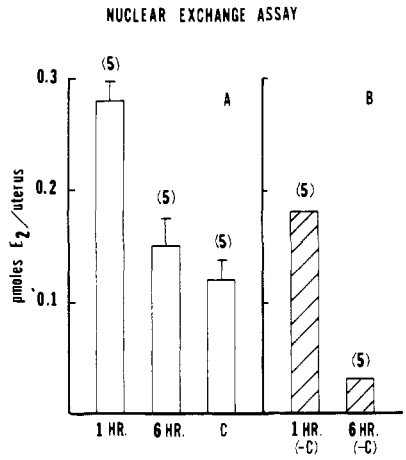

FIGURE 2: Total nuclear estradiol receptors measured by exchange assay. (A) Animals received 0.1 μg of unlabeled E_2 1 or 6 h prior to sacrifice. Control animals (C) did not receive hormone. After sacrifice, specific nuclear binding of E_2 was measured by the exchange assay (Methods and Materials). Vertical bars indicate SEM and the number of determinations is given in parentheses. Two animals were used for each determination. (B) The 1- and 6-h time points have been corrected for the control value.

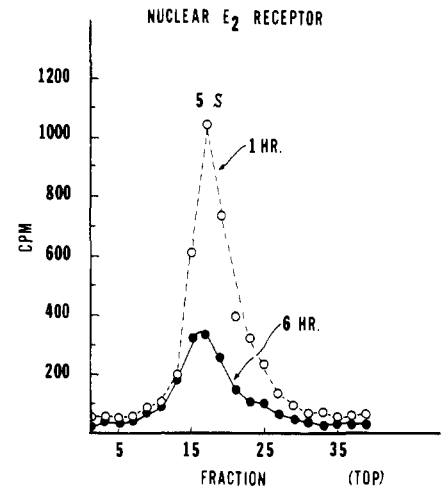

FIGURE 3: Analysis of nuclear estradiol receptors by sucrose gradient centrifugation. Groups of six animals were injected with 0.1 μg of ^3H-E_2 per animal and sacrificed at the indicated times. Nuclei were extracted with 0.4 M KCl and the extracts analyzed on 5–20% sucrose gradients as described in Methods and Materials.

These results obtained with the exchange assay (Figure 2, panel B) are similar to those obtained following direct injection of labeled estradiol (Figure 1). The amount of bound hormone measured by the exchange procedure is approximately 0.2 pmol per uterus 1 h after treatment, with a decline to 20% of this value within 6 h. These results thus suggest that the *total* number of nuclear receptor *sites* is essentially the same as the number of receptor–hormone *complexes* at 1 and 6 h after estrogen treatment.

To ensure that our values in the studies described above actually represented hormone bound to nuclear estrogen receptors, we next studied the retention patterns of the receptor by using sucrose gradients, rather than merely extracting the isolated nuclei. In these experiments animals were injected with 0.1 μg of tritiated estradiol and sacrificed 1, 3, or 6 h later. Nuclei were then isolated, washed, and extracted with 0.4 M KCl since this treatment extracts the receptor from the uterine nuclei (Shymala and Gorski, 1969; Jensen et al., 1968). The amounts of receptor–hormone complex in the nuclear extracts were then analyzed on sucrose gradients containing 0.4 M KCl. The results for the 1- and 6-h samples are illustrated in Figure 3. The 3-h sample, which sedimented in approximately the same position but with a peak height intermediate between the 1- and 6-h samples, is omitted for clarity. These peaks of radioactivity seen in the 5S region of the gradients were completely abolished if the animals were treated with 0.1 μg of tritiated estradiol plus a 100-fold excess of unlabeled estradiol.

When the areas under the three peaks obtained at 1, 3, or 6 h after hormone treatment were quantitated, it was found that the nuclei contained 0.24, 0.12, and 0.08 pmol per uterus of receptor–hormone complex, respectively. These values are in good agreement with the values illustrated in Figures 1 and 2, and again indicate that the loss of receptor–hormone complexes from the nucleus is an exponential-like process. In addition, the results illustrated in Figure 3 suggest that the receptor does not undergo any major structural change in the nucleus between 1 and 6 h after hormone treatment since the receptors extracted at various times sediment in approximately the same position.

In order to further study the possible modification of receptors in the nucleus, we examined the affinity of nuclear receptors for estradiol at various times after hormone treatment. The results of these experiments are illustrated in Figure 4. Animals were treated with 0.1 μg of unlabeled estradiol and sacrificed 1, 3, or 6 h after treatment. Nuclei were then prepared, and aliquots of nuclei from each time point were assayed by the nuclear exchange procedure at different estradiol concentrations. Nonspecific binding was measured by parallel incubations with a 100-fold excess of unlabeled diethylstilbestrol at each estradiol concentration used. The data points in Figure 4 thus represent specific estradiol binding. The results of these studies are presented in the form of a Scatchard plot (Scatchard, 1949) and illustrate that the affinity of the nuclear receptor is not appreciably changed as a function of time since the slopes of the lines are essentially parallel. The values of the dissociation constants obtained from the plots are 3.12, 2.56, and 2.46 nM for the 1-, 3-, and 6-h samples, respectively. As expected, however, the total amount of nuclear bound hormone decreases between 1 and 6 h.

The above dissociation constants are similar to the value of 1.3 nM obtained in other work using the exchange assay (Anderson et al., 1972b) and are in the general range of values obtained using other techniques (Jensen and DeSombre, 1972). While our values are thus in general agreement with those of others, more accurate values would certainly be obtained using methods with a larger number of binding sites, and greater ratios of bound/free hormone. Therefore, the observation that the values obtained at different times are essentially identical is of more significance to these particular studies than the numerical values themselves.

In addition to the studies on sedimentation profiles and binding affinities, we also sought to investigate any possible nuclear modification of estrogen receptors by examining the salt wash-out curves of the nuclear receptor at various times after hormone administration. In these studies, animals were treated with 0.1-μg doses of tritiated estradiol and sacrificed 1 or 6 h later. The nuclei were then prepared, washed and extracted with various concentrations of KCl (Figure 5). The amount of bound hormone removed by the various salt concentrations was normalized to a percent basis using the total amount of bound estradiol (i.e., ethanol

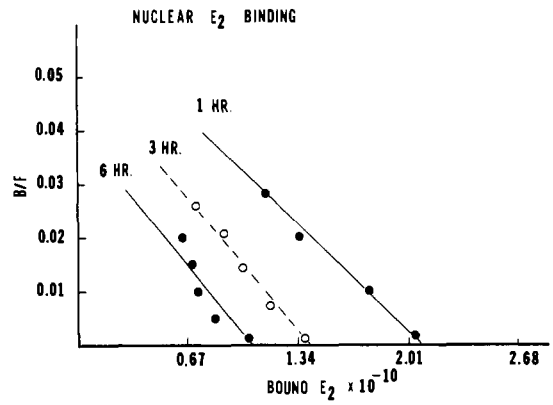

FIGURE 4: Binding of estradiol by nuclear estrogen receptor. Animals were injected with 0.1 μg of unlabeled E_2 and sacrificed at the indicated times. Nuclei were analyzed for E_2 binding by the exchange assay (Methods and Materials) at varying concentrations of ^{3}H-E_2. The results are expressed as the ratio of bound to free E_2 (B/F) vs. the amount of bound E_2 ($\times 10^{-10}$ M).

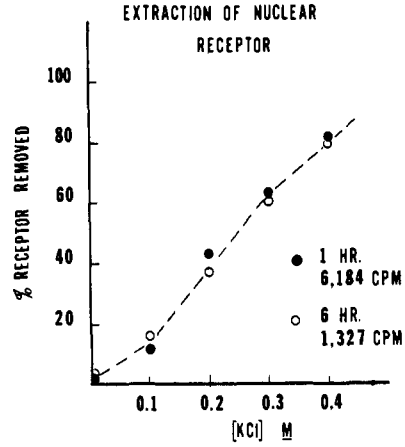

FIGURE 5: Extraction of nuclear E_2 receptors. Animals were injected with 0.1 μg of ^{3}H-E_2 and sacrificed 1 or 6 h later. The amount of receptor removed by extraction of nuclei with the indicated concentrations of KCl was determined as described in Methods and Materials. The data points represent extraction of specific binding which has been corrected for nonspecific binding by parallel experiments using uterine nuclei from animals injected with 0.1 μg of ^{3}H-E_2 plus 10 μg of unlabeled E_2.

extractable) at each time point as 100%. The 100% values were 6184 cpm and 1327 cpm per uterus for the 1- and 6-h time points, respectively. This value for the 1-h time point represents 0.21 pmol of estradiol per uterus, in good agreement with previous measurements. It is clearly seen (Figure 5) that the two wash-out curves are essentially identical which again suggests that the receptor is not modified between 1 and 6 h in uterine nuclei.

Discussion

Our initial interest in studying possible receptor modification was stimulated by a series of reports from Clark's laboratory (Anderson et al., 1972a, 1973, 1975), which pointed out a number of apparent contradictions to the "cascade" or "domino" theory of estrogen action. Such theories have postulated that a single (or limited number) of early interactions between the hormone–receptor complex and nuclear acceptor sites would trigger a chain of biochemical events that would ultimately produce *all* the estrogen-induced uterine responses. Clark's group, however, raised the possibility that estrogens worked via a "sustained input" mechanism that required a long-term (approximately 6–8 h) nuclear retention of estrogen receptor complexes. Further studies by Gorski's group also supported such a "sustained input" type of mechanism (Gorski and Raker, 1974). It has thus become increasingly difficult to explain the many effects of estrogens on the basis of a simple "cascade" mechanism.

If, as seems likely, estrogens produce their overall effects on the uterus via a sustained type of input, it is important to assess the characteristics and retention patterns of nuclear estrogen receptor complexes as a function of time after hormone treatment. These studies should help clarify the relationship between the presence of receptor–hormone complexes in the nucleus, and subsequent physiological responses, and should indicate whether nuclear receptors are modified during the course of estrogen action. Furthermore, nuclear acceptor sites are normally studied by their interactions with receptors, and a knowledge of the nature of nuclear receptors is therefore necessary to meaningfully study possible differences in nuclear acceptor sites.

Our results suggest that all estrogen-induced uterine responses are mediated by the classical "5S" nuclear estrogen receptor (Jensen et al., 1971; Giannopoulos and Gorski, 1970; Stancel et al., 1973b). This suggestion is supported by the observations that the sedimentation profiles, affinities for estradiol, and salt wash-out curves of nuclear estrogen receptors are similar at different times after hormone administration. The observation that the salt wash-out curves (Figure 5) are similar at different times after hormone administration might also suggest that the receptors are bound to similar nuclear acceptor sites at different times. However, more extensive studies are certainly required to prove or disprove this point.

The nuclear retention patterns we have observed agree with reports from several other laboratories as to the amount of hormone bound and loss of nuclear hormone–receptor complexes between 1 and 6 h after estrogen treatment (Gorski and Raker, 1974; Sarff and Gorski, 1971; Jensen and Jacobsen, 1960). The results obtained in this work from a number of different experiments utilizing several different types of measurements all agree quite well and indicate that approximately 0.2–0.3 pmol of nuclear bound estrogen per uterus are present 1 h after the administration of a "physiological" dose of hormone. At longer times after estrogen treatment there appears to be an exponential-like loss of receptor–hormone complexes from the nuclear fraction of the uterus. While the acute administration of a hormone such as estradiol, which undergoes natural fluctuations, certainly cannot be considered physiological, the 0.1-μg dose we have employed produces a maximum or near-maximum physiological response.

It is interesting to note that the results obtained by direct injection of labeled estradiol (Figure 1) are similar to the results using the exchange procedure (Figure 2). This indicates that nuclei contain few, if any, receptors with vacant hormone binding sites, in agreement with previous observations (Giannopoulos and Gorski, 1970). A likely explanation of this observation is that the hormone and the receptor are lost from the nucleus at the same rate, either separately or as a complex. On the other hand we cannot exclude the possibility that the receptor somehow becomes inactivated in the nucleus so that it cannot bind the hormone, and the hormone is then lost from the nucleus. The agreement between the results obtained by the exchange assay and by the other methods also supports the concept that the exchange

assay (Anderson et al., 1972b) accurately measures the binding of hormone to nuclear receptor sites.

The results of our retention studies differ somewhat, however, from those obtained in other studies following the injection of an identical dose (0.1 μg) of estradiol to immature animals (Anderson et al., 1972a, 1973). In these studies the amount of nuclear receptor–estrogen complexes remained essentially unchanged between 1 and 6 h after hormone treatment, although the amount observed at 1 h was quantitatively similar to the amount we observe, between 0.2 and 0.3 pmol per uterus. Initially we thought these differences might be due to differences in the route of hormone administration since we routinely administer estradiol intraperitoneally (IP) and these investigators administered the hormone subcutaneously (Anderson et al., 1972a, 1973). This does not appear to be the case, however, since we have recently determined that subcutaneous injection of tritiated estradiol yields a retention pattern that is similar to that seen following IP treatment (unpublished observations).

While we have no explanation for this apparent discrepancy at present, it is probably more important to note that all studies to date, including our own, illustrate that uterine nuclei contain significant amounts of estradiol at relatively long times after administration, e.g., 6 h. Such long-term nuclear retention of hormone is necessary for postulating a "sustained input" model of hormone action, and our retention studies would therefore be in accord with such a general model. Our results further indicate that it is the "5S" form of the estrogen receptor which is retained in an unmodified form in nuclei, and that there is only one type or one pool of this receptor present in uterine nuclei.

The major difficulty with all studies of this nature is that it has never been conclusively proved that the "5S" nuclear receptor or the observed acceptor sites of nuclear receptor binding are directly involved in mediating hormonal responses. The evidence for the role of receptors and acceptors in steroid hormone action is impressive, but circumstantial in nature (Gorski, 1973; Williams, 1974; Jensen and DeSombre, 1973; Jensen and DeSombre, 1972). Consequently, it could be that only a very small population of receptors and/or acceptors is involved in mediating hormonal responses, and these "specific" populations may be too small to observe with current techniques. While further studies are needed to investigate these possibilities, it nevertheless seems reasonable to expect that the receptors and acceptors currently being studied play a role in steroid hormone action.

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