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TWO CLASSES OF ESTROGEN RECEPTORS WHICH DIFFER IN THEIR ACTIVATION MECHANISMS

Thresia Thomas*, Benjamin S. Leung+, Winston C. Y. Yu+, and David T. Kiang*

Departments of Medicine* and Obstetrics and Gynecology+
University of Minnesota, Minneapolis, Minnesota 55455

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SUMMARY: Studies on the mechanism of activation of estrogen receptor (ER) have led us to identify two classes of receptors. Effect of molybdate on the activation of ER was determined by their affinity for nuclei or DNA-cellulose. Molybdate caused inhibition of nuclear binding with rat uterine ER, but an increase in the binding was observed with rabbit uterine ER. Similar results were observed when DNA-cellulose was used instead of nuclei. To further test the diversity in ER activation, DNA-cellulose binding of ER from 10 primary breast cancer tissues was determined in the presence or absence of molybdate. ER from 8 tissues showed inhibition of binding, one showed an increase, and one was not affected by molybdate. These results indicate the presence of two classes of ER, one whose activation is inhibited by molybdate and another whose activation is either unaffected or stimulated by molybdate. Other differences between rat and rabbit uterine ER are also discussed.

Human breast cancers with estrogen receptors are more likely to respond to hormonal therapy (1). Yet, about half the estrogen receptor-positive tumors fail to respond to such treatments (1-3). One of the reasons proposed for this inconsistency is the heterogeneity of estrogen receptors (4). Estrogen and other steroid receptors are known to consist of different molecular forms. Receptor properties that are reported to vary include molecular weight (5), sedimentation constant (6), isoelectric point (7), and differential elution during ion exchange chromatography (4). These differences prompted us to make a detailed study of the molecular characteristics of estrogen receptors from various sources and we identified two classes of estrogen receptors based on differences in activation mechanism.

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EXPERIMENTAL PROCEDURES

Materials. 17 ß-[2,4,6,7-^{3}H] estradiol ([^{3}H]-E_2) and [^{14}C] labeled bovine serum albumin (BSA) were purchased from New England Nuclear. Non-radioactive steroids, EDTA, sucrose, sodium molybdate, Dithiothreitol (DTT) and DNA-cellulose were from Sigma. Ammonium sulphate was from Schwarz/Mann.

Methods. Uterine tissue was collected from immature rats (100-150 gms.), or rabbits (4-5 lbs.). The tissue was minced and homogenized at 4°C in 10 volumes of TD buffer (40 mM Tris-HCl, 1 mM DTT) with or without 20 mM sodium molybdate. Breast cancer tissues were stored frozen in liquid nitrogen until use. Frozen tissue was pulverized before homogenization. The homogenate was centrifuged at 105,000 x g for 30 min. and the supernatant (cytosol) was collected. The cytosolic ER was labeled by incubating with 2.5 nM [^{3}H]E_2 for 2 hrs. in the presence or absence of 100-fold excess diethylstilbestrol (DES). Ammomium sulphate precipitation was carried out as described by Kon et al (8).

Nuclei were prepared as described by Muller et al (9), from rat or rabbit uteri. The tissue homogenate in TD buffer was centrifuged for 10 minutes at 1,000 x g to yield the nuclear pellet. The pellet was suspended in TD buffer and aliquots were dispensed in different tubes for binding assay. After centrifugation, the resulting pellets were washed 3 times by suspension in TD buffer, followed by centrifugation.

DNA-cellulose (5 mg. DNA/gm. cellulose) was soaked overnight in TD buffer at a concentration of 100 mg/ml. Aliquots of cytosol (200 µl) containing 100-200 fmoles of [^{3}H] ER were mixed with 200 µl of DNA-cellulose or nuclei (50-100 µg DNA) for binding assay. The assay mixtures had a final concentration of 10 mM molybdate. The reaction mixtures were incubated either at 0°C or 25°C for 90 min., with vortexing every 5-10 min. The mixtures were then centrifuged and washed twice with TD buffer. The pellets were resuspended in 2 ml. of ethanol overnight, centrifuged, and the radioactivity in the supernatant was quantitated. Results shown are representative of 2-4 experiments, carried out in triplicate.

RESULTS

Heat-induced transformation of rat uterine receptors has been well documented in the literature (10, 11). We obtained a 5.5S form of receptor by heating rat uterine cytosol at 28°C for 30 minutes (data not shown). However, a similar treatment did not change the sedimentation pattern of rabbit uterine receptors. Rabbit receptors sedimented at 4.2 ± 0.2S position, regardless of heating (Figure 1). Similar sedimentation patterns were observed for nuclear receptors extracted with 0.5 M KCl from rabbit uterine nuclei or chromatin (data not shown).

Since molybdate has been reported to prevent receptor activation (12-14), one would expect it to inhibit nuclear

binding of ER. Hence, nuclear binding of rat and rabbit uterine ER was determined in the presence or absence of molybdate (Figure 2). Molybdate caused a reduction of rat uterine ER binding to nuclei from 10% to 5% at 0°C, and from 30% to 10% at 25°C. However, with rabbit uterine ER at 25°C, molybdate caused an increase in binding from 50% to 85%. Binding at 25°C is higher than at 0°C for both tissues even though rabbit ER did not change to 5S form during heating (Figure 1). Similar results were obtained when DNA-cellulose or chromatin was used instead of nuclei (data not shown).

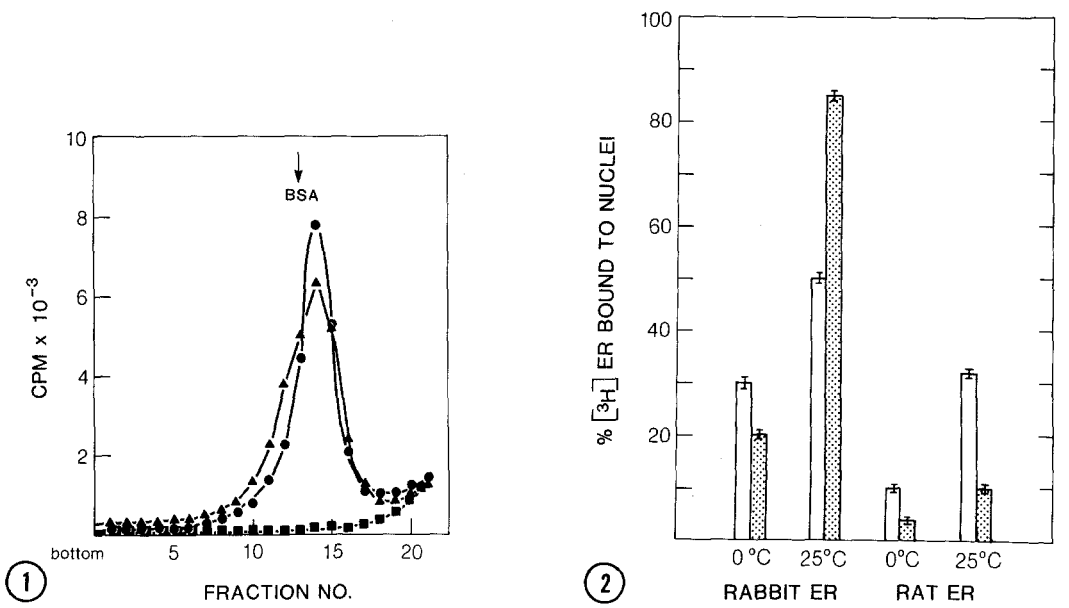

Figure 1. Sucrose gradient profile of rabbit uterine ER. Cytosol was labeled with [^{3}H]E_2 and an aliquot was heated at 28°C for 30 min. (▲—▲). Sedimentation pattern of an unheated sample (●—●) and another labeled in the presence of excess DES (■—■) are also shown. Gradients (10-30% sucrose) were prepared in buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 mM EDTA and 0.5 M KCl. Ultracentrifugation was at 257,000 x g for 16 hrs. in SW60Ti rotor. [^{14}C]BSA was used as an internal marker.

Figure 2. Effect of molybdate on the nuclear binding of rat and rabbit uterine ER. Homologous nuclei and cytosol prepared from uterine tissue were used. Binding assay was carried out as described in "Methods". Nonspecific binding to DNA-cellulose was determined in the presence of excess DES and was subtracted in each case. Results presented are bound receptor as percentage of total added. Shaded area represents presence of molybdate. The error bars represent standard deviation of triplicate samples.

Ammonium sulphate precipitation was reported to activate ER, causing a shift in the sedimentation from 4S to 5S and an increase in their affinity for DNA-cellulose at 0°C (11). Sucrose gradient analysis of rat and rabbit uterine ER is given in Figure 3A. Rat uterine ER consisted of a double peak covering 4S and 5S regions indicating partial activation. However, rabbit ER sedimented as a single peak at 4.4S. Determination of DNA-cellulose binding confirmed that rabbit ER was not activated by precipitation, since only 20% of receptors were able to bind to DNA-cellulose at 0°C. In contrast, about 50% of precipitated rat ER were able to bind to DNA cellulose

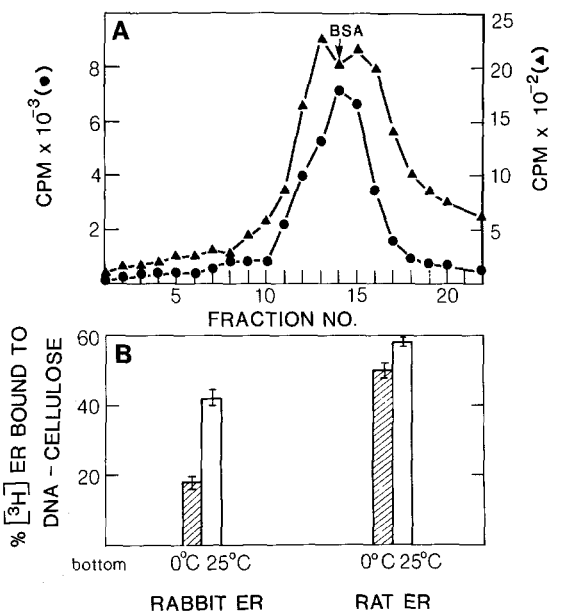

Figure 3A. Sedimentation pattern of ammonium sulphate precipitated ER from rat (▲—▲) and rabbit (●—●) uteri. Cytosol receptors were precipitated at 40% saturation of ammonium sulphate. The precipitated receptor was dissolved in TD buffer, labeled with [^{3}H]E$_2$ and subjected to sucrose gradient analysis as described in the legend to Figure 1. Only specific binding is shown in this figure.

Figure 3B. A comparison of DNA-cellulose binding of precipitated rat and rabbit uterine ER. Aliquots of receptor solution (200 µl) was mixed with DNA-cellulose and the binding was quantitated as described in "Methods". Nonspecific binding to DNA-cellulose was determined in the presence of excess DES, and was deducted. The error bars represent standard deviation in triplicate experiments.

at 0°C. Incubation at 25°C increased rat ER binding from 50% to 60%, while that of rabbit ER increased from 20% to 40%, indicating the extent of heat activation in each case.

To evaluate the significance of the different activation mechanisms with respect to ER from various sources, DNA-cellulose binding of ER from 10 primary breast carcinoma specimens were examined in the presence and absence of molybdate. As shown in Table I, the percentage of receptor binding to DNA-cellulose in the absence of molybdate varied from 11% to 45% at 25°C. When molybdate was included in the homogenization buffer binding of receptors from 8 tissues was in the range of 3% to 10%. Receptor from one tissue showed 27% binding regardless of the presence of molybdate. In another case, receptor binding increased from 13% to 21% when molybdate was included. Comparison of binding at 0°C to that of 25°C in the presence of molybdate revealed the amount of receptors that are able to undergo heat activation in spite of the inhibitory

Table I. DNA-Cellulose Binding of ER from Breast Cancer*

Ser. No.	$-MoO_4$, 25°C	$+MoO_4$, 25°C	$+MoO_4$, 0°C
1	45±0.3	4.7±0.3	2.5±0.35
2	32.8±0.4	5.95±0.5	2.95±0.05
3	28.15±0.75	8.2±0.2	2.55±0.15
4	26.2±1.5	6.6±0.1	1.65±0.15
5	21.25±0.05	2.8±0.1	1.1±0.1
6	16.75±0.05	9.15±0.55	1.65±0.15
7	14.75±0.55	4.3±0.1	1.5±0.4
8	11.1±0.6	5.45±0.45	1.15±0.05
9	27.6±1.9	27.45±1.05	7.3±0.2
10	13.2±0.1	21.05±1.25	2.5±0.2

*Data given are bound receptor as percentage of total added, with standard deviation of triplicate samples.

effect of molybdate. Thus, even in those tissues which showed inhibition of receptor binding at 25°C in the presence of molybdate, there was an increase in binding from the 1-3% level at 0°C, to the 3-10% level at 25°C.

DISCUSSION

In the steroid receptor field, the terms 'activation' and 'transformation' have been used interchangeably over the past several years (5,6,10,11). Activation is measured by an increase in the sedimentation constant of the receptor and its affinity for DNA as a result of several simple treatments such as heating, dilution, chromatography, etc. (11). Most often these two changes occur simultaneously. However, our results on rabbit uterine ER show that in this case, increase in nuclear binding due to heat activation is not accompanied by changes in sedimentation. Thus, as suggested earlier (14,15), it is more appropriate to reserve the term 'activation' for increase in nuclear binding, while a combination of changes in nuclear binding and sedimentation may be termed 'transformation'.
It is evident that variations in the activation mechanism are common so that properties such as activation of the receptor by ammonium sulphate precipitation and molybdate inhibition of nuclear binding are not applicable to all steroid receptors. Results shown in Table I indicate that the activation mechanism of a major class of receptor in 8 human breast cancer tissues resembles that of rat uterine ER. However, molybdate did not inhibit heat activation of a minor class of receptors in these tissues. The major form of receptor in the last two specimens also seem to undergo activation uninhibited by molybdate similar to rabbit uterine ER. The stimulation of ER binding to DNA-cellulose or nuclei by molybdate may be related to its stabilization effect well described for a number of steroid

receptors (11, 16, 17). Presumably both classes of receptor are functional forms since they are present in abundance in two normal animal models. However, the results in human breast cancer give evidence to the ways in which receptor properties could be different at the molecular level, and they could be reacting differently to physical and biochemical treatments in vitro and perhaps in vivo.

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