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SHORT COMMUNICATION

PRESENCE OF TWO TYPES OF ESTROGEN BINDING SITES IN MOUSE TESTIS

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Summary—The characteristics of cytosol estrogen binding sites in BALB/c mouse testis were investigated. The cytosol prepared from the whole testis contained two classes of the specific estrogen binding sites by Scatchard and Rosenthal plot analyses. The first binding site (first binder) had high affinity for 17β-estradiol (E_2; $K_d = 4.9 \times 10^{-9}$ M) and binding specificity as observed in the typical estrogen receptor. The second binding site (second binder) had lower affinity for E_2 ($K_d = 4.8 \times 10^{-8}$ M) and the binding was inhibited less vividly by diethylstilbestrol (DES) and antiestrogens in comparison with that for the first binder. Postlabeled sucrose density gradient analysis in a low salt medium revealed that the major radioactive peak of the first binder appeared at 7S region, while that of the second binder sedimented at 4S region. The 7S component showed an appreciable binding to the nuclei, while the 4S component did not show a significant binding ability to the nuclei. Much higher concentrations of the first and the second binders were found in Leydig cells preparations. These results demonstrate the presence of two types of the specific estrogen binding sites in the mouse testis especially in Leydig cells.

INTRODUCTION

For many years, it has been suggested that two types of estrogen binding sites exist in the soluble fraction of estrogen target tissues [1–3]. One site has high affinity and limited binding capacity for 17β-estradiol (E_2), and another site has lower affinity and greater binding capacity for E_2. It has been generally considered that the former site is specific binding sites for E_2, so-called estrogen receptor (ER). The putative one of the latter site which has been observed in rat uterus by Clark and his group [4], has been termed as type II site in comparison with the high affinity binding site, type I site. Subsequently, the existence of a second binding component with characteristics similar to those observed in the rat uterus has been found in many estrogen sensitive tissues such as human uterus [5], human breast cancer [6], human prostate [7], rat prostate [8], and rat liver [9].

It is well known that mouse testes possess ER, and the continuous administration of estrogen to male mice of certain strains such as BALB/c leads to the formation of Leydig cell tumors. We [10] and others [11] have been interested in clarifying the relationship between ER system and carcinogenesis in the mouse testes. Since the type II site has been reported to be correlated with growth in the rat uterus [12], we examined the characteristics of the cytosol estrogen binding sites in the mouse testis of the estrogen susceptible strain.

EXPERIMENTAL

Six to seven-week old male BALB/c mice purchased from Shizuoka Laboratory Animal Center were used. The detunicated testes were homogenized at 0–4°C in 4 vol of 0.01M Tris, 1.5 mM EDTA and 2 mM mercaptoethanol, pH 7.4 (TEM buffer) using a glass-Teflon homogenizer. Then, either cytosol or purified nuclei was prepared. The methods used were the same as reported previously [11]. The purified nuclei were intact with a little contamination of cytoplasmic tags by a phase contrast microscope. DNA recovery for the nuclear fraction was 50–60%.

The aliquots of cytosols obtained from 8–10 testes were incubated with 0.5–120 nM of $[^3H]E_2$ in the presence or absence of a 100-fold excess of unlabeled E_2 at 0°C for 4 h to allow complete equilibrium (total volume, 150 μl). To study the steroid specificity of the binding, the cytosol was incubated with either 1 or 60 nM $[^3H]E_2$ in the presence or absence of 100 times the concentrations of various unlabeled steroids at 0°C for 4 h. The amount of $[^3H]E_2$ bound to macromolecules was determined by the incubation with 25 μl of 2.5% charcoal–0.025% dextran solution for 15 min (DCC assay).

Linear 5–20% sucrose density gradients (4.6 ml) were prepared in TEM buffer. The aliquots (0.3 ml) of cytosols were applied to the top of the gradients, followed by centrifugation in a Hitachi RPS 50 rotor at 216,000 *g* for 12–14 h at 0–4°C. Fluorescent BSA (4.6S) and human γ-globulin (7.0S) were used as internal markers [13]. After centrifugation, 0.25 ml fractions were collected in tubes that contained either 1 or 60 nM $[^3H]E_2$. An identical gradient was fractionated into tubes that contained the same concentration of $[^3H]E_2$ plus a 100-fold excess of unlabeled E_2. The tubes were incubated at 0°C for 4 h. The specific estrogen binding was examined by DCC assay.

The aliquots (1.0 ml), usually consisted of 4 fractions from 7S or 4S region, were collected after a linear 5–20% sucrose density gradient centrifugation. The aliquots (0.5 ml, 0.8–1.2 mg protein) of 7S or 4S components were preincubated with 10 nM $[^3H]E_2$ in the presence or absence of 1 μM unlabeled E_2 at 0°C for 3 h and then warmed at 25°C for 30 min. These aliquots were incubated with 0.5 ml purified nuclear suspensions (approx 0.5 mg DNA) at 0°C for 1 h. After incubation, nuclei were pelleted at 1,500 *g* for 10 min. Radioactivity associated with the washed nuclei was counted.

For saturation analysis of cytosol estrogen binding sites in Leydig cell fractions, the collagenase dispersion method

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to obtain concentrated Leydig cells was carried out as previously reported [10], except for the use of TEM buffer instead of 0.01 M Tris and 1.5 mM EDTA buffer. Twenty to twenty-four testes were used, and throughout the course of these experiments these cell preparations were found to contain more than 90% viable cells based on trypan blue dye exclusion method. Total cell yield ranged between 12 and 16×10^7 with 30–40% of the cells being identifiable Leydig cells by nitro blue tetrazolium staining procedure [10].

Protein was determined by the method of Lowry [14] with BSA as standard. DNA was measured by the method of Burton [15] with calf thymus DNA as standard. [2,4,6,7-^{3}H]E_2 (100 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Unlabeled steroids were obtained from Sigma Chemical Co., St Louis, MO. Clomiphene citrate was a gift from Shionogi Co., Osaka, and tamoxifen was provided by ICI Ltd., England. All other chemicals were of analytical grade.

RESULTS

Saturation analysis over a wide range (0.5–120 nM) of [^{3}H]E_2 concentrations revealed a curve for specifically bound [^{3}H]E_2 as shown in Fig. 1A. Figure 1B shows that the curve was actually made up at least of two binding sites. The two sites could be resolved by Scatchard analysis with use of the Rosenthal methods [16] for correction of curved Scatchard plots [17]. The first binding site (first binder) in the cytosol had characteristics similar to those reported for the classical ER with a dissociation constant (K_d) of 4.9 nM and maximum binding sites (MBS) of 94 fmol/mg protein. The K_d was very close to those for cryptorchid mouse testes [11] and concentrated mouse Leydig cells [10]. The second binding site (second binder) had a lower affinity (K_d; 48 nM) and a higher binding capacity (412 fmol/mg protein) for E_2.

Furthermore, we used sucrose density gradient analysis under the low salt condition to examine these two sites. When the [^{3}H]E_2-estrogen binder complexes in the cytosol were applied to sucrose density gradient (prelabeled), considerable [^{3}H]E_2 was found to dissociate during the centrifugation periods. In the present study, therefore, each fraction obtained after sucrose density gradient analysis of unlabeled cytosol was incubated with [^{3}H]E_2, followed by DCC assay (postlabeled sucrose density gradient analysis). When 1 nM [^{3}H]E_2 was used, the major peak of estrogen binding molecules in the cytosol was found at 7S region with a lesser peak at 4S area (Fig. 2A). On the other hand, when 60 nM [^{3}H]E_2 was used, large quantities of estrogen binding molecules appeared at 4S area with less prominent peak at 7S region (Fig. 2B). Therefore, it is assumed that the 7S region is made up of the first binder and the second binder is found at the 4S region.

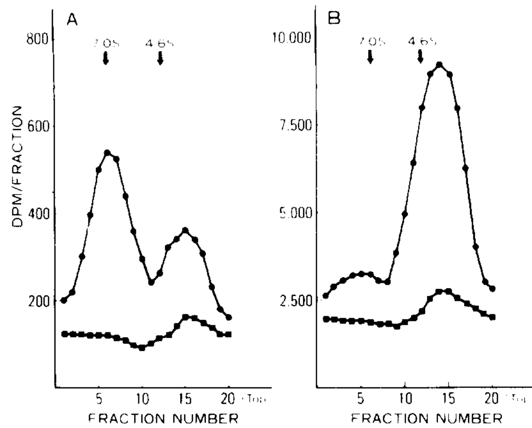

Fig. 2. Postlabeled sucrose density gradient analysis of E_2 binding sites in cytosol. The cytosol prepared from the whole testis was centrifuged as described in Experimental. Fractions were collected into tubes and incubated with a final concentration of 1 nM [^{3}H]E_2 (A) or 60 nM [^{3}H]E_2 (B) in the absence (●——●) or the presence of a 100-fold excess of unlabeled E_2 (■——■) at 0°C for 4 h, followed by DCC assay.

Table 1 shows the binding specificity of the cytosol binding sites for 1 or 60 nM [^{3}H]E_2. The binding for 1 nM [^{3}H]E_2 was inhibited markedly by both E_2 and DES. Antiestrogens, clomiphene and tamoxifen were also effective competitors. On the other hand, the binding for 60 nM [^{3}H]E_2 was markedly inhibited by 6 μM unlabeled E_2, but not evidently by DES, clomiphene or tamoxifen. Progesterone, testosterone and cortisol did not compete for binding sites for 1 or 60 nM [^{3}H]E_2. These findings suggest that the second binder in the mouse testis has binding specificity only for E_2. This result is quite different from that obtained for the first binder (Table 1).

Aliquots of either 7S or 4S fraction were collected after a linear 5–20% sucrose density gradient centrifugation, immediately preincubated with [^{3}H]E_2, and then incubated with isolated nuclei (Table 2). A large amount of specific

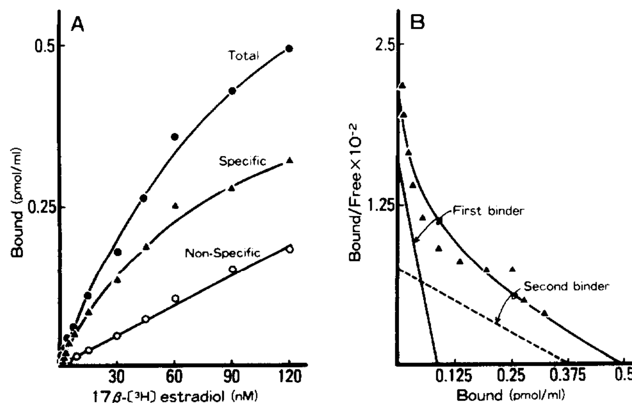

Fig. 1. Saturation analysis of estrogen binding sites in mouse testis cytosol. A, The cytosol prepared from the whole testis was incubated with various concentrations of [^{3}H]E_2 (0.5–120 nM) in the presence or absence of a 100-fold excess of unlabeled E_2 at 0°C for 4 h, followed by DCC assay. Specific binding (▲——▲) was determined by subtracting nonspecific binding (○——○) from total binding (●——●). B, Scatchard analysis of the data shown in A. The specific binding was plotted according to Scatchard. The straight lines labeled First binder (——) and Second binder (– – –) were derived by the method of Rosenthal.

Table 1. Binding specificity of E_2 binding sites in cytosol of mouse testes

Competitor	Percent bound 1 nM [^{3}H]E_2	60 nM [^{3}H]E_2
E_2	22.7 ± 3.0	36.8 ± 4.2
DES	28.5 ± 3.4	79.5 ± 3.0
Clomiphene	46.0 ± 5.1	91.5 ± 4.0
Tamoxifen	50.9 ± 6.3	92.9 ± 1.8
Progesterone	99.2 ± 6.0	100.5 ± 5.5
Testosterone	101.2 ± 5.1	104.1 ± 2.9
Cortisol	98.6 ± 5.6	101.9 ± 4.4

The cystosol prepared from the whole testis was incubated with either 1 or 60 nM [^{3}H]E_2 at 0°C for 4 h in the presence or absence of a 100-fold excess of cold competitor, followed by DCC assay. The values are expressed in percentage (mean ± SE) of [^{3}H]E_2 bound in the absence of competitor. The values were obtained by four separate experiments.

nuclear binding was observed when 7S component was used. On the other hand, specific E_2 binding sites in 4S component did not show any specific nuclear binding. Therefore, it seems that the second binder in the mouse testis does not possess nuclear binding ability.

The findings on a direct carcinogenic effect of E_2 on mouse Leydig cells [18], combined with those reported previously [10, 11], clearly indicate that ER of the mouse testis reside principally within the Leydig cells. Cell suspensions effectively concentrating Leydig cells were prepared by the collagenase digestion procedure. In the cell suspensions, 30–40% of the total cells were composed of Leydig cells. The proportion of the Leydig cells was increased by up to 10-fold, because the percentage of Leydig cells in the scrotal testis was below 5%. A saturation analysis over a wide range (0.5–120 nM) of [^{3}H] E_2 showed that two classes of the specific estrogen binding sites also existed in the Leydig cell fraction (data not shown), as were found in the whole testis. The binding analysis revealed that the K_d and MBS for the first binder were 2.2 nM and 0.35 pmol/mg protein and those for the second binder were 23 nM and 1.85 pmol/mg protein, respectively. The K_d for both the first and the second binders are close to those from the whole testis. However, MBS of both binders obtained from the Leydig cell fraction were much greater than those obtained from the whole testis, when the values were expressed on the basis of per mg protein. It was suggested, therefore, that the specific estrogen binding sites in the mouse testis exist primarily within the Leydig cells. A slight increase in the affinity for E_2 found in the Leydig cell fraction might be caused by the elimination of nonspecific binding sites present in the scrotal testis.

DISCUSSION

The present investigation demonstrates the presence of two types of estrogen binding sites in the mouse testis. The first binder has the properties of the classically described cytosol ER and the second binder has a lower affinity and a higher capacity for E_2 than the first binder. While Lin *et al.* reported ER of solely high affinity in whole rat testis [19] and ER of similar affinity in two distinct Leydig cell populations of rat [20], the confirmation of existence of two types of estrogen binding sites in mouse Leydig cells strongly suggests that the second binder is also a specific estrogen binding site in the testis.

While lower affinity sites in the mouse mammary tumor [21] has been reported to display a positive cooperativity, the second binder in the mouse testis does not display such a distinct positive cooperative binding behavior as observed in Fig. 1B. However, curvilinearities seen in the region of the second binder may represent more than two than the first and second binders.

Although these two estrogen binding sites in the cytosol of mouse testes had relatively low affinities for E_2 (4.9×10^{-9} and 4.8×10^{-8} M) compared with those (8.0×10^{-10} and 3.0×10^{-8} M) in the rat uterus [4], the sedimentation profiles of two estrogen binding sites in both tissues by postlabeled sucrose density gradient analysis were similar. In addition, high affinity estrogen binding sites appeared at the 8S region and low affinity sites appeared at the 4S region in human breast cancer [6], rat prostate [8] and rat liver [9]. It seems, therefore, that in estrogen sensitive tissues the 7-8S region on low salt sucrose gradients is made up of high affinity sites for estrogens and the 4S region is composed of low affinity sites. Since the second binder mainly formed 4S component and the 4S component did not show any nuclear binding ability, it seems that the second binder in the mouse testis does not appear to be translocated from the cytoplasm to the nucleus. In contrast with the type I site, the type II site in the rat uterus has also been shown to be non-translocatable sites [4]. It can be speculated, therefore, that the second binder may represent intermediates in the formation of the first binder or the second binder may influence the distribution of estrogens in estrogen sensitive tissues.

In contrast with the first binder, the second binder showed a much less binding affinity for DES, clomiphene or tamoxifen than for E_2 in the mouse testis. The findings are quite different from those in the rat uterus, because the type II site in the rat uterus possesses the same binding affinity for both E_2 and DES [4]. The second binder in the mouse testis, therefore, seems to be not the same as the type II site in the rat uterus. [^{3}H]E_2 binding sites in the 4S region on low salt sucrose gradient is usually attributed to α-fetoprotein in the immature rat uterus [3] and in the neonatal mouse brain [22]. However, it can be concluded that the second binder in the mouse testis is not α-fetoprotein, since α-fetoprotein could not be detected in the cytosol fraction by radioimmunoassay (data not shown).

We reported that the significant increase of ER in the BALB/c mouse Leydig cells by chronic estrogenization possibly relates to induction of Leydig cell tumor [10]. It is of great interest to study the role of the second binder in the process of carcinogenesis in the mouse Leydig cells. In

Table 2. Specific E_2 binding and nuclear binding ability of 7S and 4S components

Source	Specific E_2 binding: fmol/mg protein	Specific E_2 binding: fmol/mg DNA	Nuclear binding: % Binding
7S component	64.8 ± 11.8	33.5 ± 5.0	23.8 ± 5.1
4S component	50.0 ± 5.8	Not detectable	—

The cytosol prepared from the whole testis was applied to the top of a linear 5–20% sucrose density gradient and 7S and 4S components were collected after the centrifugation. The aliquots (0.5 ml) of 7S or 4S component (protein concentration, 0.8–1.2 mg) were preincubated with 10 nM [^{3}H]E_2 in the presence or absence of 1 μM unlabelled E_2 at 0°C for 3 h and then warmed at 25°C for 30 min. These aliquots were incubated with 0.5 ml purified nuclear suspensions (approx 0.5 mg DNA) at 0°C for 1 h. The data presented here are the mean ± SE of four separate experiments.

conclusion, we have demonstrated the presence of two classes of estrogen binding sites in the mouse testis with different characteristics. The two estrogen binding sites probably exist in the Leydig cells, but the role for the second binder with lower affinity for E_2 is not known.

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