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ESTROGEN BINDING PROTEINS IN RAT SKELETAL AND PERINEAL MUSCLES: *IN VITRO* AND *IN VIVO* STUDIES*

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SUMMARY

Specific *in vitro* binding of [^{3}H]-estradiol was demonstrated in the cytosolic fraction of levator ani/bulbocavernosus (LA/BC) muscles and thigh muscles (TM) of 50-day-old rats. Using a charcoal assay, the apparent association constant (K_a) for estradiol was found to be $1.6 \times 10^9\ M^{-1}$ in LA/BC and $0.9–2.0 \times 10^9\ M^{-1}$ in female and male TM. The K_a was not affected by 24 h of castration but the number of binding sites increased significantly ($P < 0.05$) from 7.8 ± 1.7 (SD) to 11.7 ± 2.8 (SD) fmol/mg protein in LA/BC and from 3.4 ± 1.6 (SD) to 5.8 ± 0.5 (SD) fmol/mg protein in male TM. In female TM, the level of binding sites of 24 h ovariectomized rats (4.3 ± 1.0 (SD) fmol/mg protein) was relatively similar to the one found in intact rats in diestrus phase (3.4 ± 0.8 (SD) fmol/mg protein) but higher than in proestrus phase (0.8 ± 0.15 (SD) fmol/mg protein). In both types of muscles, this cytosol estradiol binding protein sedimented in the 9–11S region of sucrose density gradients. Competition studies with androgens, progestins and corticosteroids showed that the binding was highly specific for estradiol and was only partially decreased by 5α-androstan-3β, 17β-diol.

In vivo binding of estradiol in muscles was also found after intravenous perfusion of [^{3}H]-estradiol in castrated and functionally hepatectomized rats. Specific 8–10S binding peaks on sucrose gradients were present in LA/BC and in TM cytosols. Moreover in LA/BC muscles, *in vivo* nuclear estradiol binding proteins could be detected in a 4–5S binding peak on sucrose gradients obtained with 0.4 M KCl nuclear extract. These results show unequivocally the presence of cytosol estrogen binding proteins in skeletal and perineal muscles and suggest also the existence of nuclear estrogen-binding proteins complexes in LA/BC muscles.

INTRODUCTION

While estrogen treatments have been widely used in the past to increase carcass yield of poultry and cattle, they have also been reported to depress body weight in other animal species such as adult rats [1]. In the same way, inhibitory and stimulatory effects of estrogens have been observed in guinea pig seminal vesicles. Indeed, in this tissue, estrogens induce atrophy of the epithelium and growth of the fibromuscular tissue [2]. Although the estrogen effects are very dependent upon experimental conditions, some direct effects on muscles themselves could also be suspected. According to current concepts of steroid hormone action, specific cytosolic and nuclear receptors would be required to mediate the action of estrogens in muscle cells [3].

The presence of such specific cytosol estradiol binding proteins in skeletal and perineal muscles was reported previously by our group [4]. In the present study, we have extended the analysis of some characteristics of this estradiol binding protein in LA/BC and in TM by *in vitro* and *in vivo* experiments. Furthermore, we report for the first time the presence of nuclear estradiol binding proteins in LA/BC muscles.

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EXPERIMENTAL

Chemicals. The labeled 17β-[6,7-^{3}H]-estradiol (44 Ci/mmol and 60 Ci/mmol) used in these experiments was obtained from New England Nuclear Corp, and was purified by paper chromatography before use. The radioinert steroids were purchased from Sigma.

In vitro *studies*

Animals and tissues. Adult male and female Sprague–Dawley rats (200-275 g) were obtained from le Centre de Biomédicine, Québec. Animals were used either intact or castrated. Castration was performed under ether anasthesia, 24 h before experiment, the male through a scrotal incision and the female through small lateral abdominal incisions. The female oestrus cycle was determined by daily examination of vaginal smears during one month. The proestrus,

estrus, diestrus I and II stages were identified according to Fox and Laird[5]. Only rats with regular 4 day cycles were used and they were killed between 10 and 10.30 h in the required stage of cycle. The TM and LA/BC were homogenized in respectively 5 and 10 volumes of cold 0.01 M Tris–HCl buffer, pH 7.4, containing 1.5 mM EDTA, with a Polytron PT-10 homogenizer. The homogenates were then centrifuged at 30,000 *g* for 30 min at 4°C in a Sorval centrifuge. The supernatants (cytosols) were used as the source of binding proteins.

Binding assay. Aliquots of cytosol (0.2 ml) were incubated for 1.5–3 h [incubation times long enough for equilibrium (results not shown)] with increasing concentrations (0.31–7.2 nM) of [^{3}H]-estradiol alone or in presence of 3.6×10^{-6} M of unlabeled estradiol in TM or diethylstilbestrol in LA/BC. The cytosol was treated with one volume of a suspension of dextran (0.5%), γ globulin (1%) and charcoal (5%) (DCC) for 30 min at 4°C. This suspension was centrifuged at 800 *g* for 5 min and the supernatant was dispersed in 10 ml of a toluene PPO-POPOP scintillation solution for the radioactivity determination in a Mark II (Nuclear Chicago) scintillator. The results were analysed by the method of Scatchard. The number of binding sites per cell was calculated by assuming a steroid/receptor binding ratio of 1/1 and a DNA content of 6.6 pg per diploid genome [6]. The DNA concentrations of TM and LA/BC muscles were measured according to Burton[7] with calf thymus DNA as standard.

Sucrose density gradients. Cytosol (0.5 ml) obtained from tissue homogenized in 2 volumes of buffer was incubated with 10.2 nM [^{3}H]-estradiol alone or in presence of radioinert steroid (10^{-6} M) for 2 h at 0°C. Following the incubation, the cytosol was treated with 0.2 ml of DCC at 4°C for 10 or 30 min, as indicated, in order to remove the unbound hormone. After centrifugation, 0.4 ml of the supernatant was layered on 5–20% linear sucrose gradient containing 10% glycerol. The tubes were centrifuged for 18 h at 280,000 *g* with a SW-56 or a SW-60 Ti rotor using a L2-65B Beckman centrifuge. The fractions were collected from the bottom of the tube and mixed with toluene PPO-POPOP scintillation cocktail for the radioactivity determination.

Metabolic Studies. Aliquots of cytosol (0.2 ml) were incubated with 10.2 nM [^{3}H]-estradiol for 1.5 h at 4°C. Then, the reaction was stopped by extraction with anhydrous ether; the extraction procedure was repeated three times. The [^{3}H]estradiol was separated from [^{3}H]-estrone and other [^{3}H]-polar metabolites by TLC on pre-coated silica gel plates (60-F-254-E. Merck) using chloroform–methanol (196.5:3.5, v/v) system. Unlabeled standard steroids were chromatographed along with the samples and the spots were visualized by U.V. light. The silica gel in the areas corresponding to estrone, estradiol and polar metabolites was aspirated under vacuum into small glass columns and eluted with 10 ml of methanol directly into scintillation vials. The radioactivity was determined after evaporation of the eluate.

In vivo *studies*

The experiments were done with 24 h castrated male rats (150–175 g), functionally hepatectomized according to Bruchovsky[8] prior to the infusion. In each experiment, two rats received [^{3}H]-estradiol alone and two others were perfused with [^{3}H]-estradiol in association with unlabeled estradiol; tissues of both rats were then pooled for analysis.

[^{3}H]-Estradiol (60 Ci/mmol) in saline solution containing 15% ethanol was intravenously administered (jugular vein) in the following way: a priming dose of [^{3}H]-estradiol (20 μCi) alone or in association with unlabeled estradiol (100 μg) was followed by a 1 h infusion at the rate of 2.9×10^6 d.p.m./min alone or with 6.6 μg/min of unlabeled estradiol.

At the end of the perfusion, LA/BC and TM were dissected out, rinsed three times in cold saline and homogenized respectively in 2.5 and 1 volume of buffer. The crude nuclear fractions were obtained by centrifuging the homogenates at 800 *g* for 10 min in a PR-6 International centrifuge. The cytosols were then obtained by recentrifuging the 800 *g* supernatants at 30,000 *g* for 30 min in a Sorvall centrifuge. The 800 *g* pellets were washed three times with buffer and they were first dispersed with a Potter Elvehjem homogenizer in 1.25 (LA/BC) and 0.5 (TM) volumes of Tris–HCl buffer. Then, an equal volume of 0.01 M Tris–HCl buffer containing 1 mM bacitracin and KCl was added to achieve a final concentration of 0.4 M KCl, according to the procedure of Roy and McEwen[9]. The suspensions were kept on ice for 30 min in order to extract soluble nuclear proteins. The extraction was stopped by a centrifugation at 12,000 *g* for 10 min and the supernatants were used as the 0.4 M KCl nuclear extracts.

RESULTS

Binding measurements of estradiol in male and female rat muscles

The association constant (K_a) of estradiol and the binding capacity of LA/BC and TM cytosols for this hormone were determined by a charcoal assay. Figure 1 (left panel) shows a representative saturation binding curve obtained with LA/BC cytosol. This curve was also expressed in a Scatchard plot (right panel) in order to estimate the binding affinity and the number of binding sites. The plot for B/F as a function of B estradiol yielded a straight line indicating a single class of specific binding sites for estradiol. The K_a for estradiol was 0.76×10^9 M^{-1}.

These parameters were studied in LA/BC and TM cytosols under various conditions listed in Table 1. It can be observed that the number of estradiol binding sites in LA/BC and in TM of 24 h castrated male rats is significantly higher than in intact rats. It is also worthy of note that in TM cytosol of intact

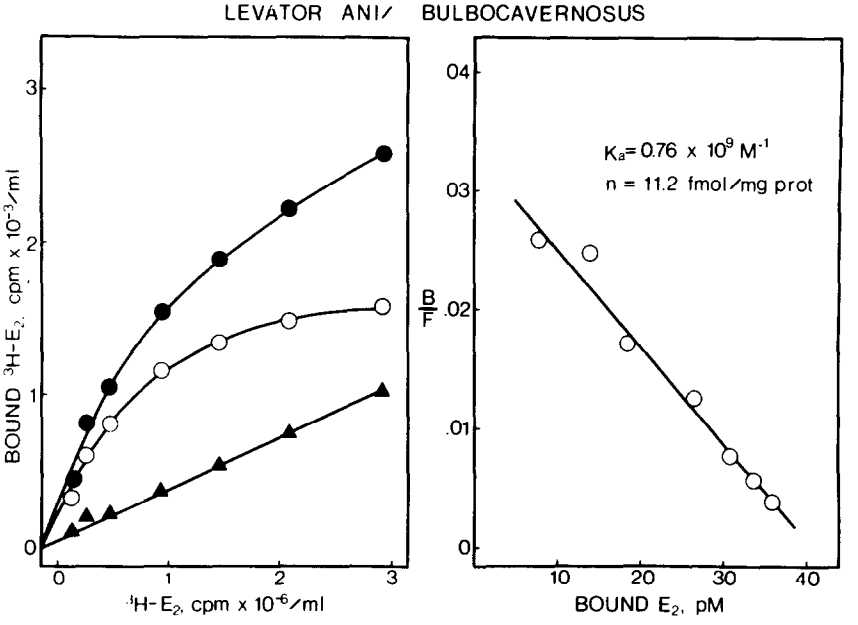

Fig. 1. Scatchard analysis (right panel) of the binding of increasing concentrations (0.31–7.2 nM) of [^{3}H]-estradiol in LA/BC cytosol of 24 h castrated male rats. Each value was corrected for the non-specific binding by incubation with an excess (10^{-6} M) of unlabeled diethylstilbestrol. ● Total binding, ▲ non-specific binding, O specific binding.

females, the level of estradiol binding sites varies during the oestrus cycle; indeed, it is found to be lower in proestrus than in diestrus. There was no significant difference between diestrus I or II stages. Moreover, values obtained during diestrus stages are not significantly different from the ones of 24 h ovariectomized rats. The results expressed in fmol per mg of protein indicate that, in LA/BC cytosol of intact or castrated rats, the number of estradiol binding sites is twice as high as in male TM cytosol. However, when the results are expressed as sites per cell we find approximately the same concentration in both muscles. On the other hand, the number of estradiol binding sites of male and female TM cytosols is comparable. Finally, under all conditions reported in Table 1, the K_a is similar and consequently not affected by sex, muscle type, castration or estrus cycle.

Studies on stability and dissociation of the [^{3}H]-estradiol-binding protein complex

The stability of the [^{3}H]-estradiol-binding protein complex at different temperatures was measured using castrated rat TM cytosol, labeled by a pre-incubation of 2 h at 4°C. Figure 2 illustrates that incubations at 15° and 25°C led to a degradation of respectively 16 and 42% after 6 h and of 32 and 73% after 20 h of incubation. Otherwise at 4°C, the complexes were very stable for at least 6 h and only 8% of degradation was observed after 20 h of incubation.

The dissociation of the [^{3}H]-estradiol-binding protein complex was measured by addition to the labeled cytosol of 3 x 10^{-6} M unlabeled estradiol. After 20 h of incubation, the following dissociation rates were observed: 58% at 4°C, 87% at 15°C and 97% at 25°C (Fig. 2).

Table 1. Estradiol binding in muscle cytosols of 65 day old male and female rats

Tissue	Rats	K_a (10^9 M^{-1})	Number of sites (fmol/mg protein)	Number of sites (sites/cell)
LA/BC	♂ 24 h castrated	0.8 ± 0.18 (5)	11.7 ± 2.8 (5)	3000
	♂ intact	1.6 ± 0.58 (6)	7.8 ± 1.7 (6)*	1800
Vastus (TM)	♂ 24 h castrated	0.8 ± 0.21 (5)	5.8 ± 0.5 (5)	3100
	♂ intact	2.0 ± 0.3 (6)	3.4 ± 0.6 (6)*	1800
Vastus (TM)	♀ 24 h ovariectomized	0.7 ± 0.2 (5)	4.3 ± 1.0 (5)	2400
	♀ intact diestrus	1.2 ± 0.5 (5)	3.4 ± 0.8 (5)	1900
	♀ intact proestrus	0.9 ± 0.1 (4)	0.8 ± 0.15 (4)†	500

Results correspond to the mean ± SD and the figures in parentheses indicate the number of determinations.
* Values are significantly lower than those of 24 h castrated rats ($P < 0.05$).
† Values are significantly lower than those of 24 h ovariectomized or intact female in diestrus stage ($P < 0.01$).

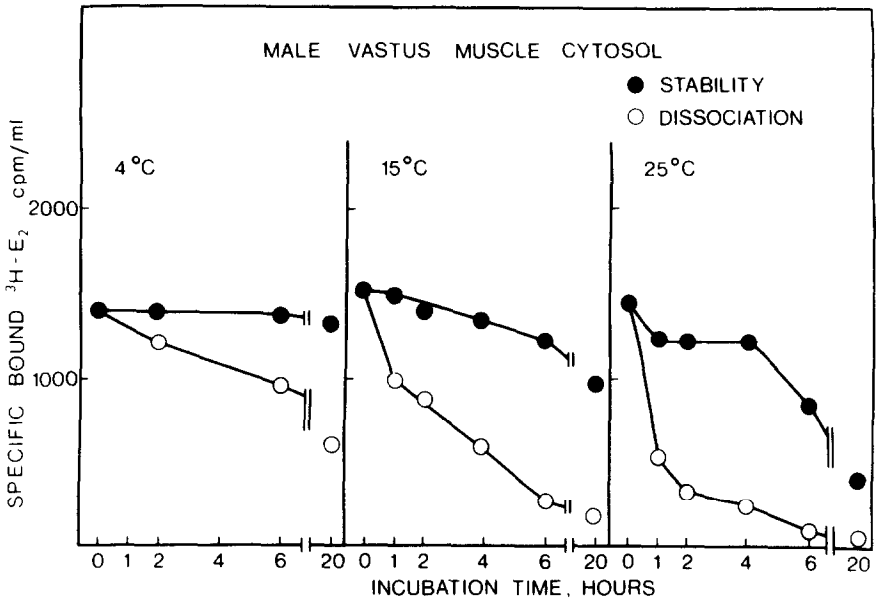

Fig. 2. Stability and dissociation of the [^{3}H]-estradiol-binding protein complex at 4°, 15° and 25°C. TM cytosol of 24 h castrated rats was labeled by an incubation of 2 h at 4°C. The stability of the complex was then measured by incubation for various times at the indicated temperature. Dissociation was measured by the addition of 3×10^{-6} M unlabeled estradiol in the labeled cytosol. Results are expressed as specific bound [^{3}H]-estradiol, determined by the DCC assay.

Characterization of the estradiol binding protein by sucrose density gradient analysis.

In a second set of experiments, the estradiol binding protein was characterized by sucrose density gradient centrifugation. Figure 3 represents typical sucrose gradient profiles of estradiol binding in LA/BC and TM cytosols of male and female rats. In both tissues of castrated or intact (not illustrated) rats, the [^{3}H]-estradiol binding is found in a 9-11S peak, saturable by addition of an excess of unlabeled estradiol or diethylstilbestrol (Fig. 3, upper and lower left panels). Another binding peak is present in the 4S region of the gradients. This peak is affected neither by estradiol nor by diethylstilbestrol in TM cytosol (Fig. 3, lower left panel). However in LA/BC, unlabeled estradiol but not diethylstilbestrol can partially inhibit the 4S binding peak (Fig. 3, upper left panel). Therefore in binding assays with LA/BC cytosol, radioinert diethylstilbestrol has been selected rather than unlabeled estradiol for the non-specific binding determination.

In two different experiments with LA/BC cytosol (Fig. 3, upper panels), we found that the amplitude of the 4S binding peak was diminished (when values were expressed for equivalent quantity of proteins applied on the gradient) by prolongation of DCC treatment from 10 min (left panel) to 30 min (right panel). The 9–11S peak is not affected by this treatment.

Competition studies of [^{3}H]-estradiol binding in diol binding protein, competitive displacement of [^{3}H]-estradiol binding by an excess (10^{-6} M) of testosterone, dihydrotestosterone, R-1881 (methyltrienolone), 5α-androstan-3β, 17β-diol, progesterone, R-5020 (17, 21-dimethyl-19-nor-pregna-4, 9 diene-3, 20-dione), cortisol and corticosterone was studied on sucrose gradients. In both, LA/BC and TM, regardless of sex and hormonal status, [^{3}H]-estradiol was not displaced by any of these hormones (testosterone is given as example in Fig. 3, lower right panel), except by 5α-androstan-3β, 17β-diol which achieved a 50% displacement of [^{3}H]-estradiol (Fig. 3 upper right panel).

Studies on in vivo *[^{3}H]-estradiol binding in muscles*

Following a 1 h perfusion of the steroid in 24 h castrated male rats, LA/BC and TM cytosols as well as 0.4 M KCl nuclear extracts were subjected to analysis on sucrose density gradients. Figure 4 shows estradiol binding profiles obtained with each fraction. In LA/BC and TM cytosols, we can observe saturable 8S binding peaks as well as large amounts of free and unspecifically bound hormone on the top of the gradients. The amplitude of the cytosolic TM peak is lower than in LA/BC cytosol although the concentration of proteins applied on the gradient was 2.5 times higher.

In LA/BC nuclear extract, [^{3}H]-estradiol binding can be detected as a peak in the 4–5S region of gradient. Such a peak is not present when an excess of radioinert estradiol is included in the perfusion

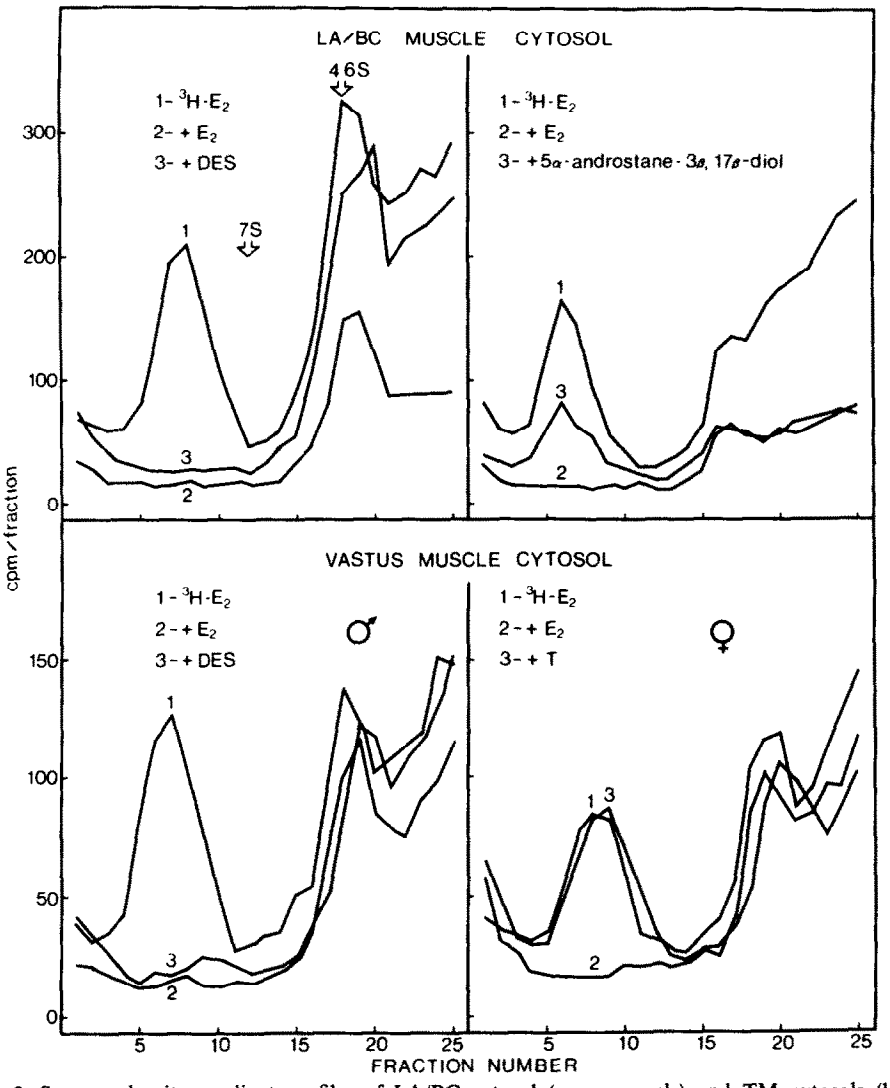

Fig. 3. Sucrose density gradient profiles of LA/BC cytosol (upper panels) and TM cytosols (lower panels) of 24 h castrated male or female rats incubated with 10.2 nM [^{3}H]estradiol alone or in presence of an excess 10^{-6} M of unlabeled steroid indicated on graph. Each aliquot of cytosol was treated 10 min with DCC before application on gradient in order to remove unbound hormone except the experiment of upper right panel where samples were treated for 30 min with DCC. The concentration of proteins applied to the gradient was for LA/BC cytosol, 8.7 mg on left panel, 6 mg on right panel, 12.5 mg for ♂ TM and 7 mg for ♀ TM. The arrows indicate the sedimentation peaks of bovine serum albumin, BSA (4.6S) and of bovine γ-globulin, BGG (7S).

Metabolic studies

Table 2 summarizes steroid composition of LA/BC and TM cytosols of 24 h castrated male rats after *in vitro* incubation or *in vivo* perfusion with [^{3}H]-estradiol. No important difference can be found between *in vitro* and *in vivo* estradiol metabolism in cytosols. However there is a small increase of polar metabolites during *in vivo* experiments. In LA/BC nuclear fraction obtained after *in vivo* estradiol perfusion, the estrone/estradiol ratio is increased when compared with cytosol; this phenomenon is not apparent in TM.

DISCUSSION

While several authors have been unable to detect the presence of estradiol cytoplasmic binding proteins in skeletal [10, 11] and in LA/BC [12] muscles, presumably for technical reasons, the data that we have presented in this study extend preliminary results from our laboratory [4] indicating the presence of specific estradiol binding proteins in rat muscles.

In muscles as well as in other target tissues [13, 14] estradiol binding proteins possess a high affinity for this hormone, a high binding stability at 4°C and a slow dissociation rate at this temperature. However, the number of estradiol binding sites appears to be relatively small when compared to the ones of rat uterus [13] and rat anterior pituitary gland [14] which may contain receptor concentrations varying between 50-1000 fmol/mg cytosolic protein. When the results are expressed as sites/cell, we observe that during oestrus cycle, TM estradiol binding (500–2000

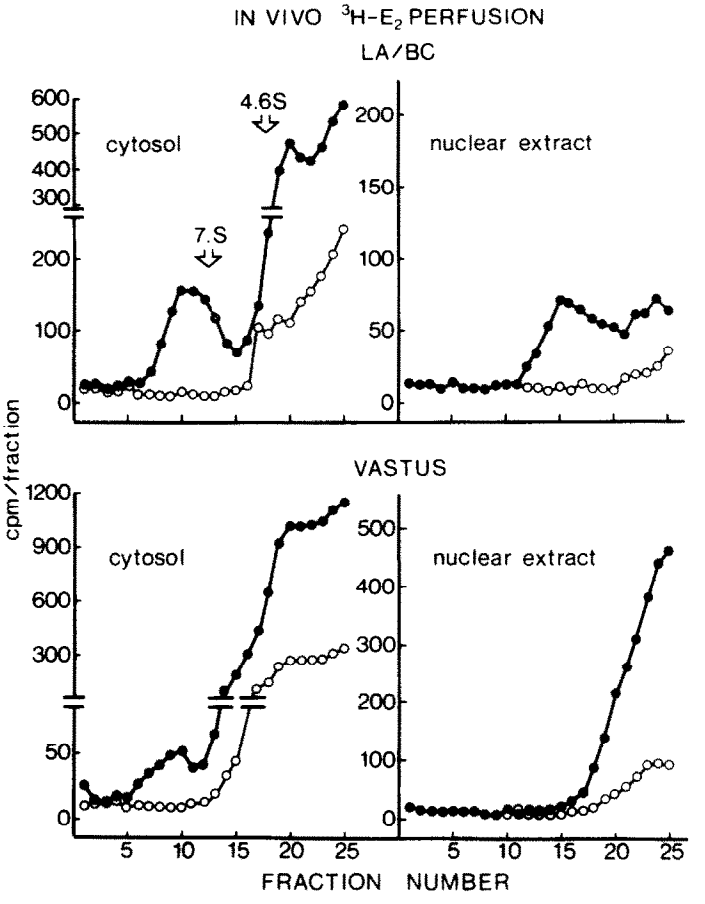

Fig. 4. Sucrose density gradient profiles of LA/BC and TM cytosol (left panels) and of LA/BC and TM 0.4 M KCl nuclear extract obtained with 24 h castrated male rats after a 1 h perfusion of [^{3}H]-estradiol alone ● or in presence of an excess of radioinert estradiol ○ (See Experimental for exact concentrations). The quantity of proteins used in each analysis was 6.5 mg for LA/BC cytosol, 2.6 mg for LA/BC nuclear extract, 16.7 mg for TM cytosol and 2.1 mg for TM nuclear extract. Sucrose gradients for nuclear extracts were prepared with 0.4 KCl in sucrose solutions.

sites/cell) represents 10–25% of uterus estradiol binding (2000–20,000 sites/cells) [15]. Nevertheless, these binding levels could be of physiological importance since they are similar to the ones of androgenic hormones [16, 17] which are known effectors of muscles cells.

A difference in measurable estradiol binding sites is observed between 24 h castrated and intact male rats. Likewise in intact female rats, the level of estradiol binding sites in muscles is found to be lower in proestrus stage, when the serum estrogen concentration is high [18], than in diestrus stages or after ovariectomy. These observations may suggest the occupation of estradiol binding proteins by endogenous hormone in intact rats but they can also indicate the depletion of cytosolic binding proteins by translocation to nucleus which is induced by high levels of serum estrogens. This phenomenon also

Table 2. Metabolism of [^{3}H] estradiol in rat muscle cytosol and nuclear fractions

Fraction	Tissue		% ^{3}H-Steroid recovered		
			Estrone	Estradiol	Polar metabolites
Cytosol	LA/BC	*in vitro**	4.4	75	0.3
		*in vivo**	11.3	70.7	5.3
	Vastus	*in vitro*	4.5	73.3	0.3
		in vivo	2.9	70.0	5.8
Nuclear fraction	LA/BC	*in vivo*	16.8	61	3.6
	Vastus	*in vivo*	5	77.5	2.3

* See Experimental.

observed in rat uterus [19] and in rat mammary tumors [20] has been particularly well documented recently by Shih and Lee[20] who demonstrated a depletion of cytosolic receptors in proestrus stage accompanied by a rise of estradiol nuclear receptors. In our experiments, exchange assays using different temperatures and incubation times do not permit to measure more estradiol binding sites in heated cytosol than in non-exchanged cytosol (results not shown). This observation is compatible with a real depletion of cytosolic binding proteins due to translocation to nucleus.

The LA/BC and TM estradiol binding proteins share another property of estradiol receptors located in rat uterus [13] anterior hypophysis [22, 24], liver [23, 24] and testis [24] namely a high specificity towards estrogens. Among all hormones studied, only 5α-androstan-3β, 17β-diol has demonstrated some affinity for estradiol binding proteins. Such a peculiarity has also been observed in other rat [25] and human [26] tissues; this competitive activity is generally explained by the simultaneous presence of two hydroxyl groups at C_3 and C_{17} β positions which confer to the molecule a significant affinity for the estrogen receptor [25, 26].

Another important physicochemical property which characterizes binding proteins (receptors) is their sedimentation coefficient on sucrose density gradient. In muscles, the cytosolic estradiol binding profiles observed on sucrose gradient after *in vitro* incubation or *in vivo* infusion are quite similar to those of rat uterus in which, for similar [^{3}H]-estradiol concentrations, two binding peaks around 8S and 4S are found [13]. However, the 4S peak can be due to a plasma contaminating protein or to a rather low affinity estrogen binding protein similar to the one found in rat preputial gland [21] since LA/BC cytosol binding in the 4S region is of lower affinity than in 9–11S region if one considers that extension of DCC treatment from 10 to 30 min diminished the amplitude of the 4S peak while the 9–11S peak was not affected.

After interaction of the hormone with its cytosolic binding site, an activation of the complex can occur which results in a conformational change of the 8S form to a 4–5S form on sucrose gradients. The activated complexes are subsequently translocated to the nuclei and can be extracted from this fraction with 0.4 M KCl [27]. By the technique used in these experiments, such a 4S soluble nuclear protein was found in LA/BC muscles but could not be clearly shown in TM.

In summary, this study demonstrates the presence in skeletal and perineal muscles of cytosolic estradiol binding proteins indistinguishable from the ones of other target tissues with regard to their affinity, their stability, their specificity and their sedimentation coefficient. Our results raise the possibility that estrogens might be able to act directly on muscular cells to influence metabolic processes and therefore carcass composition; however, further investigations should be carried out to delineate the respective role of estrogens and androgens on muscular tissue.

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