Cytosol Oestrogen Receptor of Lactating Mammary Gland

EFFECT OF HEPARIN ON THE AGGREGATION OF THE RECEPTOR AND INTERACTION OF THE RECEPTOR WITH HEPARIN-SEPHAROSE

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1. An oestrogen receptor is present in low-salt cytosol of the mammary gland of lactating mice as a large aggregate; it is excluded from gel matrix when filtered on a Sephadex G-200 column and sediments at 7S in sucrose gradients. After incubation of cytosol with heparin, the receptor is dissociated. On a Sephadex G-200 column, it is included in the gel matrix and eluted as a protein with mol.wt. 260 000 and a Stokes radius of 6.8 nm; it sediments at 6S in sucrose gradients. 2. Dissociation of the mammary-gland cytosol oestrogen receptor seems to be the result of interaction of the oestrogen-receptor complex with heparin. This receptor interacts with heparin covalently bound to Sepharose, thereafter sedimenting at 6S. By using this interaction, the cytosol receptor was purified 200-fold compared with the homogenate, with a yield of 70%. 3. The cytosol receptor that was not incubated or was incubated with heparin was much smaller during sucrose-gradient centrifugation than during gel filtration. This discrepancy can be explained by pressure-induced dissociation during high-speed centrifugation. This possibility is supported by the decrease in the sedimentation coefficient of the receptor with increased duration of centrifugation.

Mouse mammary gland binds oestradiol-17 β *in vivo* with high affinity (Bresciani & Puca, 1965; Puca & Bresciani, 1969) and oestrogen receptors have been found in the mammary gland of mice (Shyamala & Nandi, 1972; Auricchio *et al.*, 1976) and rats (Gardner & Wittliff, 1973), in breast cancer of experimental animals (Bresciani & Puca, 1965; Mobbs, 1966) and humans (Korenman & Dukes, 1970; Johanson *et al.*, 1970).

Studies on the mammary-gland cytosol receptor are still scarce because of technical difficulties when working with mammary glands; in addition to a small amount of epithelial cells in virgin glands and large amounts of fat in lactating and pregnant glands, there is a strong tendency of the receptor to aggregate.

Heparin, a naturally occurring highly sulphated glycosaminoglycan, decreases the sedimentation rate of uterine receptor centrifuged in sucrose gradients (Shyamala, 1971; Chamness & McGuire, 1972).

In the present paper the effect of heparin on oestrogen receptor from mammary-gland cytosol of lactating mice was studied. Our results show that oestrogen receptor in low-salt cytosol is present as a large aggregate, from which it is dissociated by incubation with heparin. This dissociation seems to be the result of interaction of oestrogen receptor

with heparin, as shown by the binding of receptor to heparin-Sepharose. This interaction allows, in a single step, a 200-fold purification of the mammary-gland receptor.

Apparent discrepancies between data obtained by gel filtration and those obtained by sucrose-gradient centrifugation are discussed in terms of pressure-induced dissociation of the receptor during sucrose-gradient centrifugation (Baierlein & Infante, 1974).

Experimental

Materials

Reagent-grade materials were used in all experiments. Horse heart cytochrome *c*, horse muscle myoglobin, bovine plasma albumin and bovine immunoglobulin G were from Serva, Heidelberg, Germany.

Dithiothreitol was from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex G-25 (coarse grade) and G-200, Blue Dextran 2000 and Sepharose 4B were from Pharmacia Fine Chemicals, Uppsala, Sweden. [2,4,6,7-³H]Oestradiol-17 β (85 Ci/mmol) and [³H]-leucine (30 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Heparin was from Fisher, Fair Lawn, NJ, U.S.A. (sp. activity 144 i.u./

mg) or from Fluka A.G., Buchs, Switzerland (sp. activity 140 i.u./mg).

Buffers

The following buffer solutions were used: 10 mM-Tris/HCl, pH 7.4 (T buffer); 10 mM-Tris/HCl, pH 7.4, containing 1 mM-dithiothreitol (TD buffer).

Sucrose-gradient centrifugation

Sucrose gradients (5–20%, w/v) in a total volume of 4.2 ml were used. Centrifugation was carried out in a Beckman Spinco L2-65 ultracentrifuge at 2°C for 14 h at 45000 rev./min unless specified otherwise. The sample was labelled with [³H]oestradiol-17 β , and treated with charcoal before centrifugation. Bovine plasma albumin (4.45S) was used as a reference for computing the sedimentation coefficients of the receptor.

Gel filtration

A standard Pharmacia column K16/40 with an upward-flow adaptor was used. The upward flows were generated by peristaltic pumps. The void volumes (V_0) of Sephadex G-200 columns were measured by using Blue Dextran 2000, and the total volumes (V_t) by using [³H]leucine. All operations were carried out at 4°C by using the following reference proteins (molecular weights and Stokes radii, in parentheses, are from Andrews, 1970): cytochrome *c* (12400; 1.64 nm), myoglobin (17800; 2.07 nm), plasma albumin (67000; 3.55 nm), immunoglobulin G (160000; 5.3 nm).

Oestrogen-binding activity

Oestrogen-binding activity was measured as previously described (Auricchio *et al.*, 1976).

Radioactivity assay

Radioactivity was measured in a Nuclear-Chicago liquid-scintillation counter, by standard procedures. Either a mixture containing 2.66 g of 2,5-diphenyloxazole/l, 33 mg of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/l and 30% Triton X-100 in toluene, or Instagel, from Packard Instruments Co., Downers Grove, IL, U.S.A., was used, according to the volume of the sample. Details of the assay have been described previously (Auricchio *et al.*, 1976).

Heparin-Sepharose preparation

For this, 2 mg of heparin/ml of 0.1 M-NaHCO₃, pH 8.5, was coupled to CNBr-activated Sepharose 4B (300 mg of CNBr/ml of packed gel) as described by Cuatrecasas (1970) (1 g of activated Sepharose was used per ml of heparin solution).

Preparation of cytosol

The preparation was performed as described previously (Auricchio *et al.*, 1978). Cytosol was

labelled by incubation with 5 pM-[³H]oestradiol-17 β for 30 min at 4°C. Checks of binding specificity were carried out in parallel trials, by decreasing the specific radioactivity of [³H]oestradiol by addition of a 150-fold excess of unlabelled oestradiol-17 β . Cytosol prepared as described above contains 6–7 mg of protein/ml. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine plasma albumin as standard.

Results

Gel filtration

As shown by gel-filtration experiments, an oestrogen receptor from the mammary gland of lactating mice is present as a large aggregate in a low-salt concentration cytosol.

In fact, specific binding activity for oestradiol-17 β is eluted from a calibrated Sephadex G-200 column at the V_0 of the column (Fig. 1a).

Incubation of cytosol at 0°C for 30 min with 6 mg of heparin/ml shifts most of the oestrogen-binding activity from V_0 of the Sephadex G-200 column to the elution volume (V_e) of a smaller protein (Fig. 1b). The molecular weight of this protein measured

Fig. 1. Effect of heparin on gel filtration on Sephadex G-200 columns in TD-buffer of [³H]oestradiol-labelled mammary cytosol, or (NH₄)₂SO₄ precipitate of cytosol. Cytosol was labelled with [³H]oestradiol (sp. radioactivity 85 Ci/mmol) and, subsequently, filtered through a Sephadex column (a), incubated at 0°C with 6 mg of heparin/ml for 30 min and thereafter filtered through a Sephadex column (b) or incubated with heparin as in (b) with the addition of 40%-satd. (NH₄)₂SO₄ (c), and the precipitate was redissolved in TD buffer and filtered through a Sephadex column. Total volume of the columns was 51 ml. Arrows indicate the peak of Blue Dextran (V_0). The effluent was monitored for radioactivity. To check the specificity of binding activity eluted from the column, parallel filtrations (not shown) were performed with cytosol treated as in (a), (b) and (c) except for labelling of cytosol with lower-specific-radioactivity [³H]oestradiol-17 β (sp. radioactivity 0.57 Ci/mmol); no measurable binding activity was eluted.

Fig. 2. Effect of heparin on sucrose-gradient centrifugation of $[^3\text{H}]$ oestradiol-labelled cytosol in TD buffer

In (a), cytosol was labelled by incubation with $[^3\text{H}]$ oestradiol (sp. radioactivity 85 Ci/mmol) before layering on the sucrose gradient; in (b), cytosol was incubated at 0°C with 6 mg of heparin/ml for 30 min after labelling performed as in (a) and layered on the sucrose gradient. To check the specificity of binding activity sedimenting in sucrose gradients, parallel gradients (not shown) were run with cytosol treated as in (a) and (b) except for labelling cytosol with lower-specific-radioactivity $[^3\text{H}]$ oestradiol-17 β (sp. radioactivity 0.57 Ci/mmol); no measurable sedimenting binding activity was observed. Centrifugation of the 5–20% (w/v) sucrose gradients was in a Beckman L2-65 ultracentrifuge (rotor SW60) at 45000 rev./min for 14 h at 2°C ; fraction volume, 0.2 ml. Arrows indicate the peak of the reference protein, bovine plasma albumin (4.45 S).

Fig. 3. Dependence of the sedimentation coefficient of cytosol oestrogen receptor labelled with $[^3\text{H}]$ oestradiol on the duration of centrifugation

Cytosol was labelled by incubation with $[^3\text{H}]$ oestradiol (sp. radioactivity 85 Ci/mmol) and layered on sucrose gradients in TD buffer. Centrifugation of the 5–20% (w/v) sucrose gradients was in a Beckman L2-65 ultracentrifuge (rotor SW60) at 39000 rev./min at 2°C ; fraction volume, 0.2 ml.

Fig. 4. Interaction of cytosol oestrogen receptor with heparin-Sepharose

$[^3\text{H}]$ Oestradiol-labelled cytosol was incubated with heparin-Sepharose in the presence of 5 mM-MgCl₂ at 0°C . The gel was poured into a column (2 ml) and extensively washed with TD buffer containing 0.15 M-KCl until neither radioactivity nor A_{280} was measurable in the washing buffer. At this point binding activity was eluted from the gel with TD buffer containing 3 mg of heparin/ml (a). To check the specificity of binding activity eluted from heparin-Sepharose, a parallel experiment (not shown) was performed with cytosol treated in the same way, except for the labelling of cytosol with lower-specific-radioactivity $[^3\text{H}]$ oestradiol-17 β (sp. radioactivity 0.57 Ci/mmol); no activity was eluted from the gel by heparin. Then 0.3 ml of the sample containing the binding activity eluted from heparin-Sepharose was run through sucrose gradients (b). Centrifugation of the 5–20% (w/v) sucrose gradients was in a Beckman L2-65 ultracentrifuge (rotor SW60) at 45000 rev./min for 14 h at 2°C ; fraction volume, 0.2 ml. The arrow indicates the peak of the reference protein, bovine plasma albumin (4.45 S).

by the method of Andrews (1964) is 260000 and the Stokes radius measured by the method Porath (1963) is 6.8 nm. A similar shift is observed after salting out with $(\text{NH}_4)_2\text{SO}_4$ when cytosol was pretreated with 6 mg of heparin/ml (Fig. 1c).

Sucrose-gradient centrifugation

The receptor centrifuged through sucrose density gradients in our standard experimental conditions (45000 rev./min for 14 h) sediments in the 7S region (Fig. 2a). After incubation of cytosol at 0°C with

6mg of heparin/ml, the sedimentation coefficient of binding activity is decreased to 6S (Fig. 2b).

In a different type of experiment, [^3H]oestradiol-labelled cytosol was centrifuged in sucrose density gradients at 39000 rev./min for 6, 10, 14 and 20h and the sedimentation coefficients were computed by using serum albumin samples as references. The results are reported in Fig. 3. Increasing the centrifugation time drastically decreases the sedimentation coefficient, from 9.7S after 6h centrifugation to 6.3S after 20h.

Interaction with heparin-Sephadex

A solution containing 5mM-MgCl₂, 1mM-EDTA and 10mM-KCl was added to [^3H]oestradiol-labelled cytosol (5ml); the cytosol was treated with charcoal and incubated with 1g of heparin-Sephadex. The gel was stirred for 1h at 0°C, poured into a column (2ml) and washed with TD buffer containing 0.15M-KCl until neither radioactivity nor A_{280} was measurable in the washing buffer. [^3H]Oestradiol was eluted with 3ml of TD buffer containing 3mg of heparin/ml (Fig. 4a). The experiments described below show that the eluted radioactivity was bound to the receptor: it was eluted at the V_0 of a 20ml Sephadex G-25 column; the binding activity was destroyed by heating for 5min at 45°C (Auricchio *et al.*, 1976); no [^3H]oestradiol was eluted from the heparin-Sephadex when cytosol was labelled with lower-specific-radioactivity [^3H]oestradiol-17 β (sp. radioactivity 0.57Ci/mmol). The eluted receptor, when centrifuged through sucrose gradients in standard conditions, sedimented at 6S (Fig. 4b).

The receptor, after elution from heparin-Sephadex, was purified about 200-fold compared with the homogenate with a yield of 70%.

Discussion

The oestrogen receptor in cytosol of low ionic strength of lactating mammary gland is largely aggregated, since it is eluted at the V_0 of a Sephadex G-200 column. Neither the nature nor the role of this aggregate is clarified by our experiments.

Treatment of cytosol with heparin dissociates the oestrogen-binding activity from the aggregate into a receptor with mol.wt. 260000 and a Stokes radius of 6.8nm. This molecular form of the receptor is stable, since it still occurs after (NH₄)₂SO₄ precipitation, which is known to greatly favour aggregation, when cytosol was pretreated with heparin. This receptor sediments at 6S by sucrose-gradient centrifugation, whereas the receptor of cytosol, not incubated with heparin, sediments in the 7S region. The receptor present in heparin-treated cytosol seems to be in a still partially aggregated form; in fact, in the presence of NaBr, a chaotropic salt inhibiting interaction of the receptor with other molecules,

the mammary cytosol receptor shows values of Stokes radius and sedimentation coefficient (3.7nm 4.2S respectively; Auricchio *et al.*, 1978) smaller than those observed after incubation of cytosol with heparin.

The heparin-induced dissociation of the receptor could be due either to the removal of molecule(s) associated with the receptor or to the direct interaction of the receptor with heparin. Several proteins are known to interact with heparin, as well as with heparin covalently bound to Sepharose (Rosenberg & Damus, 1973; Waldman *et al.*, 1975; Iverius & Östlund-Lindqvist, 1976). More recently a similar interaction has also been reported for a uterine cytosol receptor (Molinari *et al.*, 1977). The mammary-gland cytosol receptor tightly binds to heparin-Sephadex, from which it is eluted as a form sedimenting at 6S. This fact both suggests that direct interaction between receptor and heparin is responsible for receptor dissociation and confirms the strong similarity between the properties of the mammary-gland receptor and those of the uterine oestrogen receptor (Auricchio *et al.*, 1978), and also offers a powerful tool to purify the mammary cytosol receptor. Our experiments do not, however, clarify the mechanism by which interaction of the receptor with heparin causes receptor dissociation.

Several lines of evidences suggest that, during sucrose-gradient centrifugation, the oestrogen receptor is dissociated by hydrostatic pressure: specific binding activity is present in cytosol as an aggregate with a molecular weight of at least 400000 (it is eluted at the V_0 of a Sephadex G-200 column); it sediments in sucrose gradients as a single peak of an apparently smaller protein (7S); similarly the heparin-treated receptor has mol.wt. 260000 as determined by gel filtration through Sephadex G-200, whereas it sediments at 6S. Pressure-induced dissociation is also a possible explanation of the observed decrease in sedimentation coefficient of the receptor with the increase of centrifugation time (Baierlein & Infante, 1974). The strong dependence of the sedimentation coefficient on the centrifugation conditions should be taken into adequate consideration when physical parameters of steroid receptor are measured with this technique.

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