Supplement

Dimeric Structure of Unoccupied Activated Estrogen Receptor

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Data reported at the fifth international CBT symposium on intracellular hormone receptors and ligands-dependent transcriptional regulation. Huddinge, Sweden, 1993. (Reference 60)

Objective of the study

Interaction of the native receptor (R)* with an estrogenic steroid causes its dimerization. This "activation" is evident by the conversion of the native 4S receptor in its activated 5S form when sedimented in a high-salt sucrose gradient. To determine whether this activated R retains its 5S dimeric structure in the absence the hormone, we removed it by a dextran-coated charcoal treatment (DCC) before centrifugation. Tritiated estrone (*E1) was used for that purpose because its complexion with ER is less stable than with estradiol (*E2); * E2 was used for a subsequent labeling of the" stripped" receptor.

Experimental protocol

Immature rat uteri were homogenized in 10mM Tris-HCl pH 8.5 buffer (T10) at 0° C. R activation was then achieved by two successive incubations of the homogenate with 20nM *E1, at respectively 0° C (30 min) and 28 °C (75 min). Harvested nuclei were then removed of residual cytosolic R (Rc) by three or four successive washes (radioactivity of washes was measured to monitor cytosol removal). Activated nuclear complex (*E1-Rn) was finally obtained by an extraction of the nuclei (1 hour at 0° C in 500 mM KCl) followed by an ultracentrifugation (1 hour at 100,000 g). Cytosolic *E1Rc was clarified according a same ultracentrifugation.

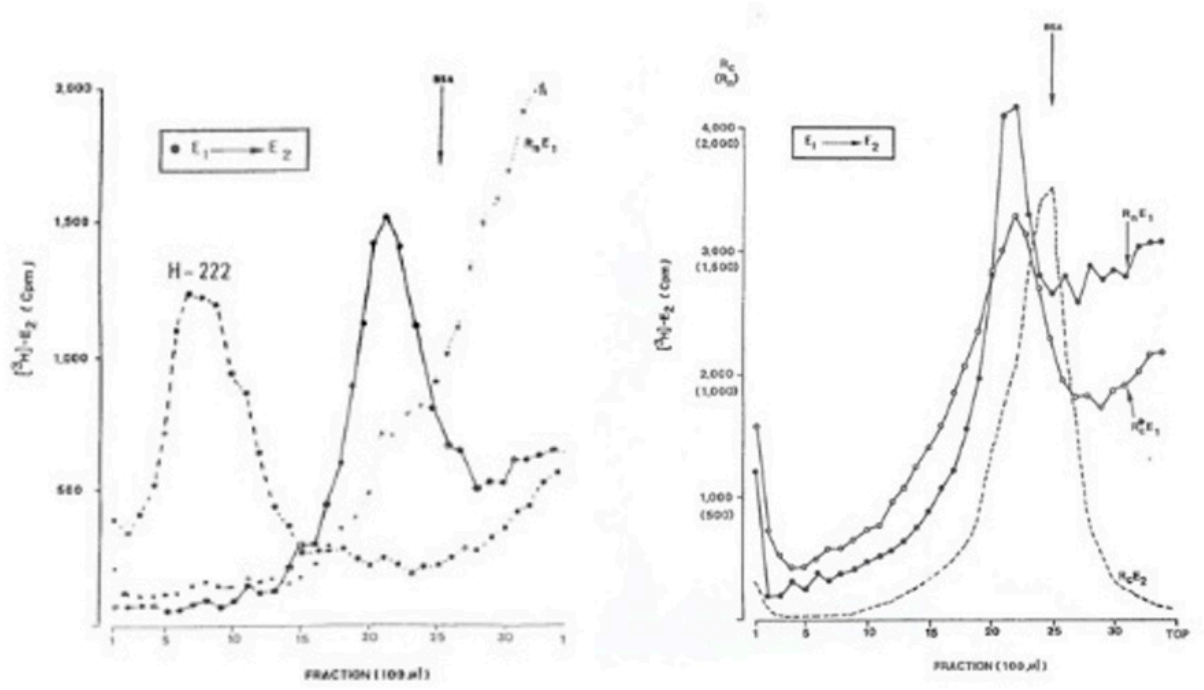
Activated *E1-nR complex was identified by sucrose gradient sedimentation (10-30% sucrose in T10 containing 500mM KCl and 1.5 mM EDTA; 50,000rpm, Beckman SW60 Rotor). This complex dissociating partly during sedimentation (**#1**), an exchange of bound *E1 was achieved with *E2 prior centrifugation (1 hour with 2nM *E2 at 0° C, removal of unbound* E2 by a 15 min treatment with DCC). Sedimentation patterns of these radio-labeled complexes were compared with the migration of Bovine Serum Albumin (BSA) run in parallel (4.4 S).

*E1 was totally removed from the *E1Rn complex by a DCC treatment (1 hour at 35 °C). Maintenance of the dimeric structure of this "stripped" receptor was assessed by an 1 hour incubation at 0° C before sedimentation with *E2 or * CME2 [125-iodine(*Z*-17a-(2-iodo)-11b-vinyl)-11b-chloro methyl estradiol) , a ligand with an exceptional high binding affinity (Quivy J. et *al. J. Steroid* Molec. *Biol*.**1996**, 59, 103-117). Measurement of Rn levels in each fraction of the sucrose gradient with the Abbott ER-EIA was another identification approach.

Such identification procedures were also applied for 5S ER dimeric form present in the cytosol to assess whether it precedes or depends of ERE binding.

Results

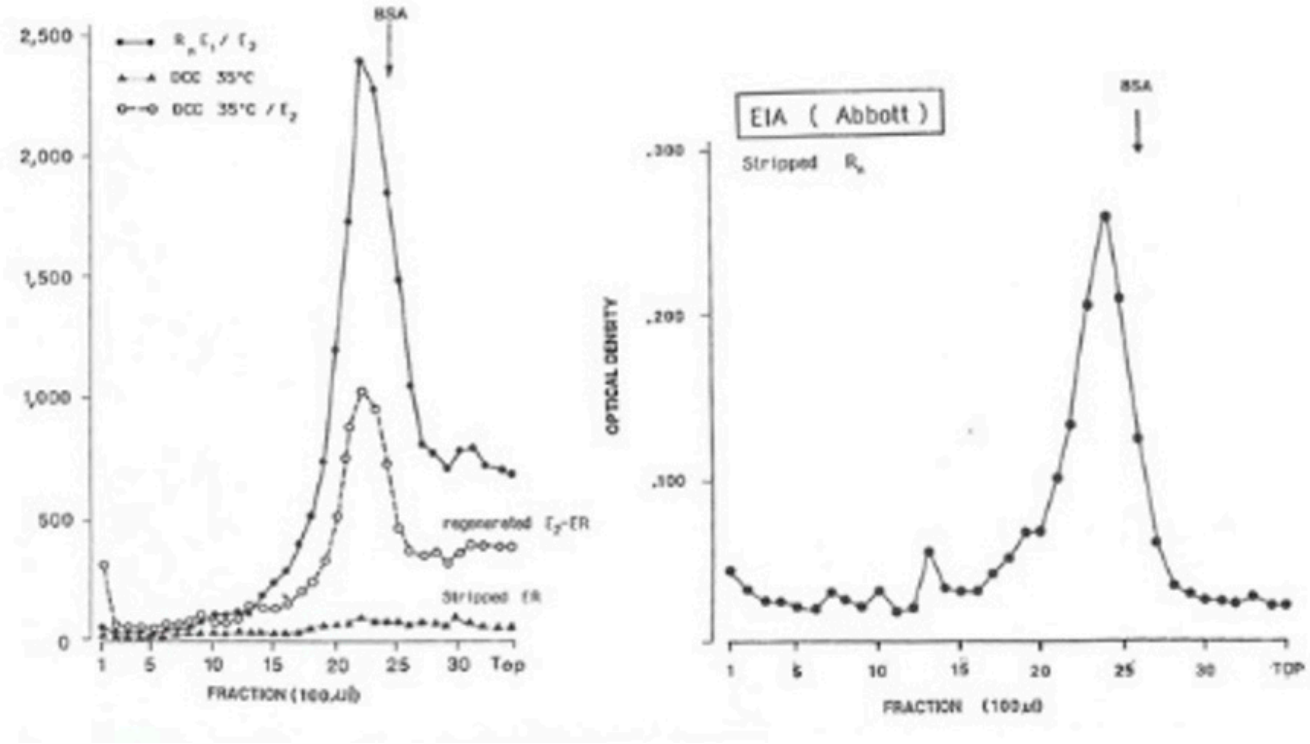
1. Requirement of E1* / E2* exchange for the identification of the nuclear E1*-induced 5S dimeric



Left. Rn*E1 complex was extremely unstable during sedimentation giving rise to an absence labeled peak. Exchange of *E1 by *E2 before sedimentation palliated this property: a 5S peak (migration in front of BSA, 4.4 S) was indeed easily detectable. Shift of this peak towards the bottom of the sucrose gradient by a pretreatment of this sample with the H-222 anti-R monoclonal antibody proved the specificity of this peak.

Right. E1*/E2* exchange was also efficient for the cytosolic 5S Rc*E1 complex (Rn *E1 and Rc*E1 migrate at the same position in sucrose gradient). A control non heat-activated cytosolic Rc*E2 migrated indeed at the position of BSA, as a 4S complex. Interestingly, this Rc*E2 complex gave a symmetrical peak without any evidence of dissociation during sedimentation in contrast to the 5S *E2 exchanged Rc*E1 and Rn*E1 complexes for which a partial dissociation was recorded. Hence, a decrease of estrogen binding affinity occurred during the 4S/5S transition.

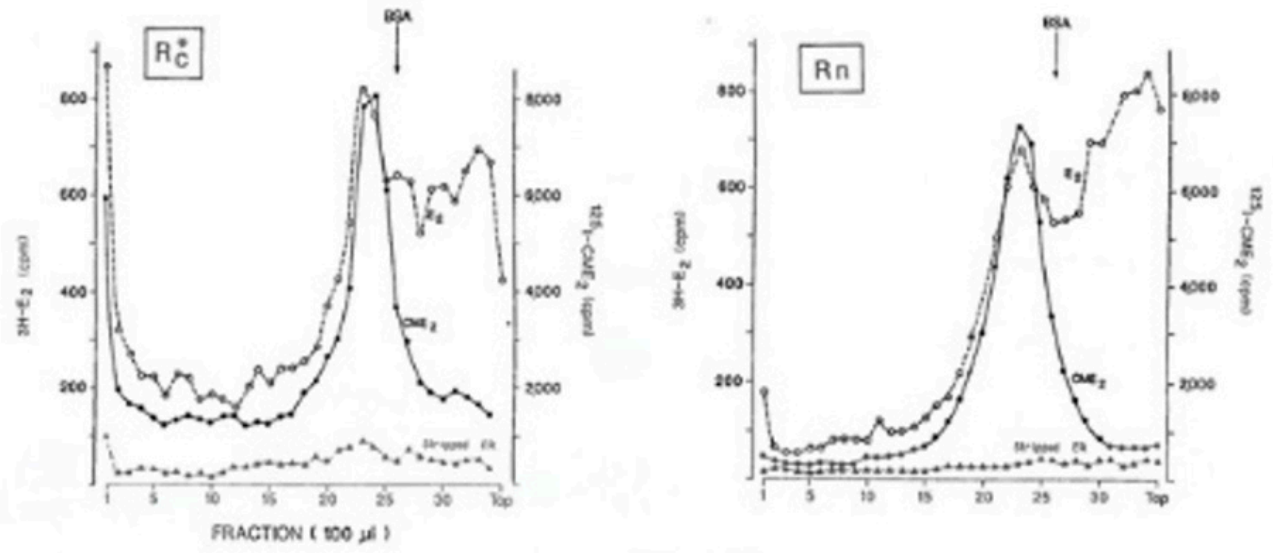
2. Extraction of E1* from RnE1* and RcE1* complexes fail to affect their 5S dimeric form



Left. Sedimentation of the "stripped Rn" gave no peak indicating a total extraction of bound *E1. Labeling of this receptor with *E2 failed to totally restore the binding recorded before extraction suggesting some structural change of the estrogen binding pocket. Stripped activated Rc behaved similarly. All tentative to palliate this decrease of *E2 binding were unsuccessful [addition to the samples: sodium molybdate (10mM) aprotinin (50 µg/ml; addition to the sucrose gradient buffer: 10% glycerol or10mM thioglycerol, replacement of sucrose by glycerol (5-35%)], suggesting that this decrease was a consequence of the dimerization.

Right. Receptor level measurement in each fraction of the sucrose gradient by the Abbott Enzyme Immuno Assay gave solely a 5S entity confirming its stability, even after steroid extraction.

3. Labeling of the stripped dimeric receptor with CME2 proves a lack major alteration of the estrogen binding pocket of the receptor to explain its weak estrogen binding ability



Labeling of stripped R*c (***Left***) and Rn (***Right***) with *CME2 (an E2 derivative of which association with the receptor in aqueous solution is almost irreversible) gave a symmetric 5S peak without any dissociation tendency during centrifugation, in contrast with the *E2 labeling. This difference suggests that the weak binding affinity of the stripped dimeric receptor form results from a subtle change of its estrogen binding pocket which occurs in the cytoplasm at the time of acquiring a capacity to move to the nuclear chromatin for ERE binding.

Conclusion

Estrogen-induced dimerization of the estrogen receptor (4S-5S shift in sucrose gradient sedimentation) is irreversible. A loss of estrogen binding affinity occurs in parallel. Underlying procedures seem already occur within the cytoplasm suggesting that they may contribute to the nuclear transfer of the receptor to the nucleus and related ERE association.

Complementary note. (R) : experiments having been performed before the identification of two estrogen receptors, R was used instead of ERα, as in the original report.*