FORMATION OF ESTROGEN NUCLEAR RECEPTOR IN UTERUS : EFFECT OF ANDROGENS, ESTRONE AND NAFOXIDINE.

H. ROCHEFORT, F. LIGNON and F. CAPONY

Groupe de Recherches sur la Biochimie des Stéroïdes - I.N.S.E.R.M - U.58 -
Institut de Biologie - Faculté de Médecine
34 - Montpellier - France -

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SUMMARY

Different steroids and nafoxidine were tested in vitro on rat uteri for their ability to transfer the estrogen cytosol receptor (Rc) to the nucleus. This transfer was evaluated by testing the simultaneous decrease of the number of specific binding sites for estradiol in cytosol and increase of the same binding sites in nuclear extracts. Three classes of compounds could be distinguished according to their affinity for Rc and activity to transfer Rc to the nucleus ; those which bind and transfer Rc (estradiol, estrone and nafoxidine), those which neither bind nor transfer Rc (progesterone, cortisol) and finally those which do not bind Rc, but transfer it to the nucleus (androgens). It is suggested that androgens modify membrane permeability for the estrogen cytosol receptor.

Estradiol, after entry into a target cell, is first bound to its specific cytosol receptor (Rc), and then translocated to the nucleus where it is retained with its binding protein (I) (2). This transfer of the hormone to the nucleus which was observed after administration of ^{3}H estradiol in vivo or in vitro might be of interest in explaining the effect of estradiol on nuclear RNA transcription (3). Both cytosol and nuclear receptor(s) for estradiol have similar properties (4) (5) and estradiol in vivo induces a simultaneous decrease of Rc and increase of the nuclear receptor (6). Therefore it is generally assumed that the nuclear receptor is either a part or the complete cytosol receptor, which has been translocated to the nucleus after its interaction with the hormone (2) (6) (7).

In this work, the specificity of different compounds in transfering Rc to uterine nuclei was tested, by measuring their in vitro effect on the number of specific estrogen binding sites, in cytosol and

nuclear extracts. Androgens, estrone and nafoxidine were shown to imitate this particular estradiol effect to some extent.

METHODS :

Uteri from prepuberal Wistar rats (three weeks old) were incubated for I h at 37°C in an Eagle medium with O_2/CO_2 95/5 according to Giannopoulos and Gorski (8), in the presence or absence of different steroids. 6-7 ^3H estradiol (SA = 48 C/mM), 6-7 ^3H estrone (SA = 45 C/mM) and I-2 ^3H testosterone (SA = 46 C/mM) were from CEA, the androgens[1], progesterone, estradiol, cortisol, 98% pure were given by Roussel-Uclaf (France) and nafoxidine (U. II,IOO) was a gift of Dr O. Kadruka (Upjohn Company). The following steps were performed at O-4°C. After homogenization of the tissue in tris HCl pH 7.4 buffer containing EDTA I.5 mM and centrifugation at IO5,000 g x 90 min., cytosol and pellet were obtained (5). The KCl 0.5 M extract of this pellet was defined as the "nuclear" extracts, since control experiments performed with purified nuclei gave similar results as when using the IO5,000 g pellet.

Measurement of specific binding sites for estradiol were performed after saturation of sites by ^3H estradiol (5 nM) followed by adsorption of the unbound ligand on dextran coated charcoal (9). Steroids and nafoxidine were 98% pure as checked by thin layer chromatography (TLC) on silica gel F 254 unsing benzene-ethyl acetate 3 : 2 V/V. Metabolism of tritiated hormone during uteri incubation was also tested by TLC after extraction of tissue by ether-ethanol (4/I). Counting of radio-activity was performed in a toluene scintillation mixture with 25% efficiency, quenching being corrected by external standard. Sucrose gradient centrifugation of nuclear extracts was performed in Tris KCl medium at O-4°C as indicated previously (5).

RESULTS :

I°) - Choice of the experimental system :

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1 The following compounds were used.

Testosterone (T) : I7 β -hydroxy-androsta-4ène-3-one
Dihydrosterone (DHT) : I7 β hydroxy-5 α androsta-3-one
3 β Androstanediol (3 β Adiol) : 5 α Androstane 3 β , I7 β diol
Dehydroépiandrosterone (DHA) : 3 β hydroxy androsta 5-ène-I7-one
Epitestosterone (TI7α) ; I7 α hydroxy androsta-4-ène-3-one
Progesterone (P) : Pregna-4-ène-3-20-dione
Cortisol (F) : II β , I7 α , 2I -trihydroxy pregn-4-ene-3,20-dione.
Estradiol (E_2) Estra-I,3,5-triène 3, I7 β diol
Estrone (E_I) = 3-hydroxy-estra-I,3,5 triène-I7-one

In order to define the activity of a drug on the transfer of Rc to the nucleus, the specifically bound estradiol was simultaneously measured in cytosol and nuclear extracts, after in vitro administration of the drug (7). In previous experiments, a cell free reconstituted system, made of nuclei incubated at 25°C for 20 min. with cytosol prelabelled by ^{3}H estradiol was tested. Under these conditions, estradiol was found to slightly increase the number of nuclear receptor sites (Fig.Ib) but not to modify the number of the cytosol estradiol binding sites (Fig. Ia). These results can be explained by the simultaneous occurence of two effects of estradiol on its receptor, one being the protection of the binding sites against thermo-inactivation (5) and the other, the translocation of Rc to the nucleus. The fact that the amount of cytosol receptor was constant in this cell free system suggested that the transfer and the protective activities of estradiol were approx. the same. Since the results obtained with the whole tissue system were found to be similar to those obtained in vivo, as previously described by others (8), we have decided to test the transfer activity of different drugs using this in vitro technique.

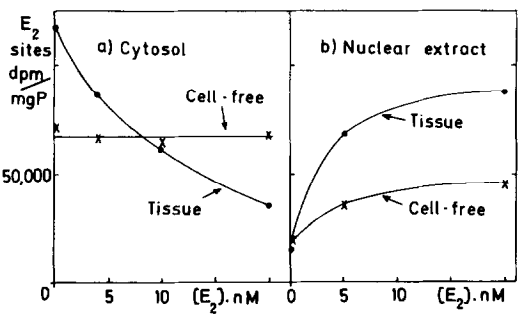

FIG. I

Comparison between cell free and tissue experimental procedures for testing estradiol efficiency in the transfer of Rc to nuclei.

I5 prepuberal rat uteri were either incubated I H. 37°C with increasing concentration of ^{3}H estradiol in Eagle medium (Tissue) (●) or homogenized in Tris EDTA, and then incubated with estradiol at 25°C for 20 min. (cell free) (x). The incubation time corresponded to a maximum transfer in both conditions. The estradiol binding sites of cytosol (a) and "nuclear" extract (b) were tested as described in Methods.

2°) - Effect of compounds which bind to the estradiol cytosol receptor.

After incubation of uteri with increasing concentrations of ^{3}H estrone or ^{3}H estradiol, the estrogen binding sites were measured by the charcoal technique, as indicated in Methods. It was verified that

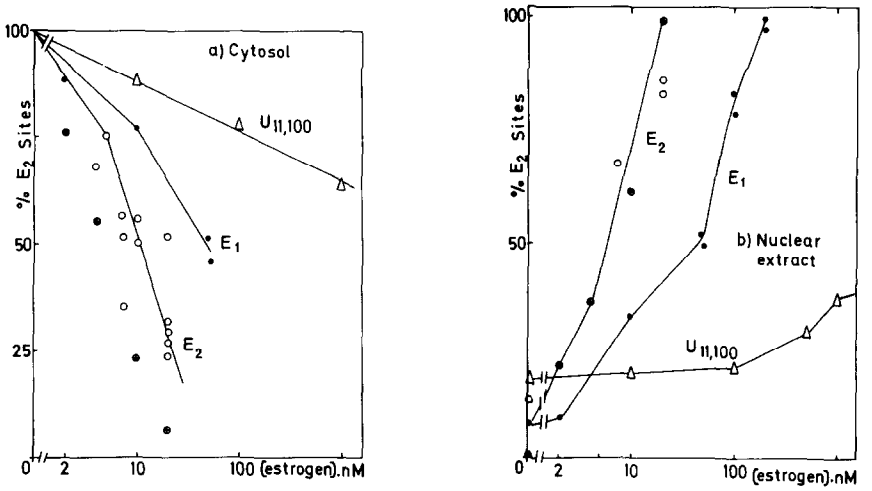

FIG. 2

Effect of estrogen and antiestrogen on the formation of estradiol nuclear receptor.

Uteri were incubated with increasing concentrations of estradiol (E_2), estrone (E_1) or nafoxidine (U. II,100), then the specifically bound estradiol per mg of protein, in cytosol and "nuclear" extract, were tested as indicated in Methods.
a) Number of specific estradiol binding sites in cytosol : Results are in percent of maximum number of sites (100% = 150,000 dpm/mg protein).
b) Number of specific estradiol binding sites in the "nuclear" extract (100% = 200,000 dpm/mg protein).
The results obtained with estradiol by Gianopoulos and Gorski (8) are represented for comparison (⊕). The cytosol and nuclear sites measured after incubation of uteri with nafoxidine (△) have been corrected assuming a 50% thermo-inactivation.

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the use of ^{3}H estrone or ^{3}H estradiol was equally appropriate for the charcoal technique. Estrone, as estradiol, was found to be efficient both in decreasing cytosol binding sites (Fig. 2a) and increasing nuclear binding sites (Fig. 2b). However the concentration of estrone (50 nM) necessary to reach the 50% effect was about 7 times more than that of estradiol. The metabolism of ^{3}H estrone into ^{3}H estradiol in incubated uteri, checked by TLC of the homogenate extract, was less than 5 per cent. It is thus unlikely that this transformation could explain the whole effect of estrone in the formation of the nuclear receptor. After incubation of uteri with ^{3}H estrone, sucrose gradient centrifugation of the nuclear extract displayed a radioactive 4-5 S complex similar to that obtained with ^{3}H estradiol (Fig.3).

Nafoxidine, which is an anti-estrogen capable of binding to

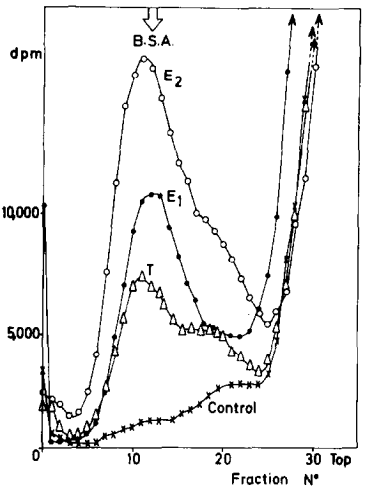

FIG. 3

Sucrose gradient pattern of the estrogen nuclear receptor formed after in vitro treatment by estrone or testosterone.

Uteri were incubated with ^{3}H estrone (E_1 - 200 nM), ^{3}H estradiol (E_2 - 20 nM), non radioactive testosterone (T - IμM) or no ligand (control). The T and control "nuclear" extracts were subsequently saturated with ^{3}H estradiol 5 nM. Then the 4 "nuclear" extracts were centrifuged at 2°C in a sucrose gradient of 5-20% containing KCl 0.5 M. Bovine serum albumin (BSA = 4.6 S) was run separately as a standard.

Rc with an affinity of about I/30 that of estradiol (KD ≃ 7 nM) (9), was also tested for its ability to transfer Rc to the nucleus. Since this compound is known to occupy estradiol binding sites, these sites had to be liberated before labelling with ^{3}H estradiol. For this purpose the cytosol and nuclear extract were incubated at 25°C and 45°C respectively with a charcoal dextran suspension. After centrifugation of the charcoal, the supernatant was cooled and incubated with ^{3}H estradiol (5 nM) for 60 min. This treatment allowed a complete dissociation of the nafoxidine nuclear receptor complex, but 30 to 70% inactivation of this binding protein was also observed. The specifically bound estradiol was then tested by the charcoal technique or by sucrose gradient centrifugation as indicated above. Nafoxidine was shown to decrease the cytosol receptor sites of estradiol (Fig. 2a) and to increase the nuclear receptor sites of estradiol (Fig. 2b). However, its minimum effective concentration required (approx 500 nM), was much higher than when using estradiol or estrone and in this case a complete transfer was not observed. No marked difference could be detected between the sedimentation constants of the nuclear receptor obtained after incubation cf either estradiol or nafoxidine (Fig. 4).

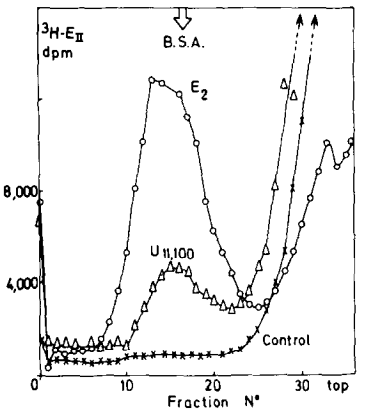

FIG. 4

Sucrose gradient pattern of the estrogen nuclear receptor formed after in vitro treatment by nafoxidine.

Uteri were incubated in the presence of nafoxidine 2 μ M (U II,100), 3H estradiol 20 nM (E_2) or no ligand (control). Then, the "nuclear" extracts were treated by charcoal suspension at 45°C, and reincubated with a saturating concentration (5 nM) of ^{3}H estradiol. These extract were thereafter analysed in a sucrose gradient buffered with Tris KCl 0.5 M at 0°C. BSA (4.6S) was run separately.

3°) - Effect or compounds which did not bind to the estradiol cytosol receptor.

The "transfer activity" of other steroids, which did not compete with estradiol for Rc, were tested using the same methodology. Since these steroids did not bind to the cytosol receptor, the estradiol binding sites were easily measured by simply adding a saturating concentration (5 nM) of ^{3}H estradiol to the cytosol and nuclear extracts. Two series of compounds could be distinguished (Fig. 5). Progesterone and cortisol were both without effect on the receptor sites in cytosol, as well as those in nuclear extracts. However it was surprising to find that the androgen compounds were active in this respect. According to their relative efficiency (shown in Fig. 5a and b), they could be classified as follows : Dihydrotestosterone and testosterone $>$ androsta 4ene dione and 3β androstanediol $>$ Dehydroepiandrosterone and testosterone I7α . Sucrose gradient centrifugation of the nuclear extract, obtained after incubation of uteri with testosterone I μ M, displayed the same pattern as when using estradiol in the incubation medium. It had been checked by TLC that testosterone was not metabolised into estradiol by uteri during this incubation procedure. In any case, the aromatisation

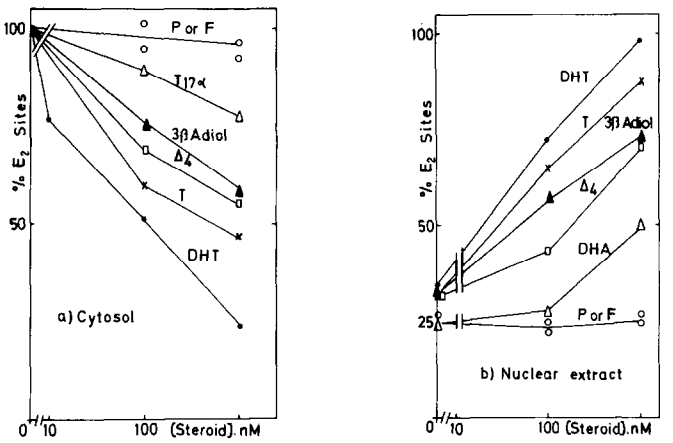

FIG. 5

Effect of androgens on the formation of estradiol nuclear receptor.

Non radioactive C_{19} and C_{21} steroids were incubated with uteri as indicated above. Cytosol and nuclear extracts sites, were then saturated with ^3H estradiol 5 nM and the estradiol binding sites were measured by the charcoal technique.

a) Number of specific estradiol binding sites in cytosol (100% = 150,000 dpm/mg protein)

b) Number of specific estradiol binding sites in "nuclear" extracts (100% = 200,000 dpm/mg protein).

The results with testosterone and dihydrostestosterone are the mean of three experiments.

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of non radioactive testosterone into estradiol would have prevented the labelling of nuclear sites by ^3H estradiol. Since testosterone increased the amount of nuclear sites available for ^3H estradiol, it was concluded that the effect found with testosterone could not be due to its transformation into estrogen. Also, no inhibition of estradiol binding to Rc was detected with testosterone at concentrations of up to 1 μM. Therefore, it was infered that some compounds could induce the transfer of estrogen cytosol receptor to the nucleus without binding to the estradiol sites of this protein. According to results shown in Fig. 5, the relative "transfer activity" of testosterone and dihydrotestosterone, as compared to that of estradiol, was approx 15 times less. The maximum effect was about the same for both androgens and estrogens but it was reached with higher concentrations of the former than of the latter.

DISCUSSION :

Even though no definite conclusion can be drawn from comparing the in vitro effect of a drug on a receptor, to its in vivo biological

activity, the transfer activity of estradiol, estrone, nafoxidine and androgens can be compared, on one hand to their affinity for the estrogen receptor to be transfered, and on the other hand to their uterotrophic activity.

A good parallel was found between affinity, transfer activity and biological activity for estrone and estradiol ; especially since estrone was also found to be active in vitro, in transfering Rc to the nucleus. However, higher concentrations of estrone were necessary than when using estradiol ; this could explain the negative results obtained by others (I0). Even for high concentrations (5 μ M), the antiestrogen nafoxidine, contrary to estradiol, had a partial effect on the translocation of Rc to the nucleus. On the other hand, nafoxidine at the same concentration had been shown to completely inhibit estradiol binding to Rc. The fact that nafoxidine is a partial agonist (II) might be explained by its partial effect on the transfer of Rc to the nucleus, and its antiestrogenic activity might be due to a complete displacement of estradiol from its binding sites.(9).

A marked discrepancy between binding and transfer activities was shown with androgens which did not bind to Rc but were active in transfering Rc to the nucleus. This effect of androgens might be related to their uterotrophic and antiestrogenic activities observed after in vivo administration of non physiological doses ($\simeq$ I2 mg) of testosterone (I2). Moreover, the androgen induced transfer of Rc to the nucleus raises the question of the mechanism of formation of the estradiol nuclear receptor. It is generally assumed that estradiol binding to Rc is necessary for the transfer of this protein to the nucleus. This assumption is not favored by the present work, since testosterone which did not bind to the estradiol sites of Rc, was shown to be efficient in inducing its translocation to the nucleus. It is suggested that the C_{19} steroids induce the estrogen nuclear receptor formation through interaction with some components other than the cytosol estradiol receptor. These components could be membranes, whose permeability for Rc would be modified by the presence of androgens. Since a specific androgen cytosol receptor has been described in uterus (I3, I4), it is asked whether this protein interferes during the androgen induced translocation of the estradiol cytosol receptor to the nucleus. Whether the nuclear receptors formed after androgen or estrogen treatment of uteri are identical is unknown, even though many of their properties are similar (in preparation). Their mechanism of formation could also be quite diffe-

rent, however we suggest that estrogens like androgens, might modify membrane permeability. This hypothesis is supported by two observations ; first, that in the cell free experimental system containing mainly lysed nuclei, estradiol has a very poor transfer activity (see Fig.I), second that the binding of Rc to the uterine chromatin or DNA does not seem to be estrogen dependent since it also occurs in the absence of steroids. (J. André and H. Rochefort in preparation). Therefore, the following working hypothesis is proposed that estradiol provokes the entry of Rc into the nucleus but does not favor its binding to chromatin components.

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