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Relations Between ^{3}H-Estradiol Uptake and Receptor Content of Estrogen Responsive Tissues of Castrated Female Rat

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The time course of ^{3}H-Estradiol-17 β (^{3}H-E_2) uptake, and estrogen receptor content in estrogen responsive tissues were studied between 0 and 12 h after injection of 0.5 μg/kg of ^{3}H-E_2 or cold E_2 injection to castrated adult female rats.

The plasma concentration of ^{3}H-E_2 between 10 min and 2 h after injection was in the range of the plasma E_2 level of cyclic rat. The total ^{3}H-E_2 uptake was well correlated with the receptor content in all tissues. The rank order of ^{3}H-E_2 uptake was: uterus (Ut) > anterior pituitary (Ap) > hypothalamus (Ht) > plasma. The cytosol ^{3}H-E_2 uptake showed its maximal level 10 min after injection in all tissues. Parallel time course between plasma ^{3}H-E_2 and cytosol uptake was obtained for each separate tissue. The nuclear ^{3}H-E_2 uptake showed its maximal values 2 h after injection with a subsequent decline.

Cytosolic estrogen receptor (Rc) content showed a depletion-replenishment cycle after cold E_2 injection in all tissues. Nuclear estrogen receptor (Rn) content in Ut increased progressively from 0 to 14 h after injection, but in Ap it showed its maximal level 2 h after injection, declining afterwards.

In Ap, nuclear ^{3}H-E_2 uptake and Rn level showed parallel time courses. The maximal level of both parameters coinciding with the time of maximal Rc depletion. However, the Rn level in Ut increases more slowly at greater length than the nuclear ^{3}H-E_2 uptake, both processes being divergent. These findings are interpreted as the expression of tissular differences in the rate of nuclear receptor formation from the Rc-E complex previously translocated into nucleus and attached to chromatin.

Key words: ^{3}H-estradiol uptake, Estrogen receptor, Cytosolic receptor.

The existence of specific macromolecular receptors in the estrogen responsive tissues, that bind the estrogens with high specificity, has been well established (11, 16). When estrogen enters the cell it forms a complex with its cytosolic receptor (Rc), before translocating into the nucleus, where it attaches itself to the genomic apparatus and influences cellular activity (23).

The most relevant events about these mechanisms, have been demonstrated by means of cell fractionation procedures and ^{3}H-E_2 exchange assays (1, 12, 18, 20), as well as by means of *in vivo* administration of tracer doses of ^{3}H-E_2 proceeding either to an autoradiographic study for radioactivity distribution, or to measuring the radioactivity accumulated by tissue fractionation (11, 19, 30).

The results obtained by these methods were often in disagreement, many contradictory arguments having been offered to explain the differences away (12, 28).

In an attempt to understand the causes of these discrepancies, the present paper describes a method that allows the study of the time course relationship between ^{3}H-E_2 uptake and the simultaneous fluctuations of cytosolic and nuclear estrogen receptors in target tissues. It is essentially based on the quantification of ^{3}H-E_2 uptake, and the receptor content of tissues after the injection of separate single doses of 0.5 μg of ^{3}H-E_2 or cold E_2.

Materials and Methods

Animals. Adult female Sprague-Dawley rats (200-250 g wt.) from our own colony, were maintained under 12 h light and 12 h darkness, with water and food *ad libitum.* Ovariectomy was performed under ether anesthetic two or three weeks before experimentation.

^{3}H-E_2 uptake. The method described by MCEWEN *et al.* (19) was used with minor modifications. A ^{3}H-E_2 (3, 4, 6, 7-(^{3}H)-β-estradiol S. A. 100 Ci/mMol. The Radiochemical Centre) solution of 0.5 μg/ml in saline solution containing 5 % of ethanol, was injected intraperitoneally at a dose of 0.5 μg/kg. At a given time (0.2 to 12 h), the rats were anesthetized and sacrificed by bleeding. A blood sample was collected and residual blood was removed by 50 ml of saline injection in the left ventricle.

Uterus, anterior pituitary and hypothalamus (limited anteriorly by 1 mm before the optic chiasma, laterally by the hypothalamic fissures, posteriorly by a line just behind the mammilary bodies, and 2 or 3 mm in depth) were quickly dissected, weighed and homogenized in ice cold TE buffer (10 mM tris-HCl, 1.5 mM EDTA-Na_2 and pH 7.4), in an all glass Potter-Elvehjem homogenizer, at 0-4° C and 200-250 rpm with intervals for cooling. The homogenate was centrifuged at 850 *g* for 15 min and then the supernatant was centrifuged again at 105,000 *g* to obtain the cytosol. An aliquot of cytosol was mixed vigorously with scintillation cocktail (0.5 % PPO, and 0.025 % POPOP in toluene), and counted for radioactivity in a Packard 3390 spectrometer with a 38 % efficiency.

The 850 g pellet was washed three times with excess of TE buffer and the last pellet extracted with 1 ml of toluene. An aliquot was mixed with the scintillation cocktail and counted for radioactivity.

Experiments with cold estradiol. A solution of 0.5 μg/kg of 17-β-estradiol (Merck) per ml was prepared in saline solution containing 5 % of ethanol, and injected intraperitoneally at dose of 0.5 μg/kg of body weight. The tissue fractionation was performed as described for ^{3}H-E_2 experiments.

Cytosolic estrogen receptor assay. The method described by MESTER *et al.* (20) and MESTER and BAULIEU (21) was used with minor modifications. In short, aliquots of cytosol (100, 200 or 500 μl for Ut, Ap or Ht respectively) were incubated in triplicate with 10 nM ^{3}H-E_2 alone or with 2 μM of diethylstilbestrol (Sigma) at 30° C for 30 min. A suspension of dextran-coated charcoal (0.05 % and 0.5 % respectively) was added, and incubated again for 10 min. After centrifugation an aliquot of supernatant was mixed with scintillation cocktail, and counted for radioactivity with a 37 % efficiency.

Nuclear estrogen receptor assay. The method described by ANDERSON *et al.* (1) was followed with minor modifications. Briefly, the 850 g pellet of the tissue fractionation, was washed three times with an excess of TE buffer, and the last pellet resuspended in 2.5 ml of TE buffer. Aliquots of this crude nuclear suspension were incubated in triplicate with 10 nM ^{3}-H-E_2, alone or with 2 μM of diethylstilbestrol for 30 min at 37° C. Excess of ^{3}H-E_2 was removed with 2 ml of TE buffer. Radioactivity was extracted from nuclear pellet with 1 ml of toluene, and an aliquot mixed with the scintillation cocktail and counted for radioactivity at 37 % efficiency.

Statistical procedures. Comparison between groups was performed by means of the Student *t* test.

Results and Discussion

The time course for ^{3}H-E_2 amounts in plasma and for cytosol of Ut, Ap and Ht from castrated female rats injected with 0.5 μg/kg of b. wt. is shown in fig. 1. The ^{3}H-E_2 level in plasma and cytosol in all the assayed tissues increases 10 min after ^{3}H-E_2 injection, and declines progressively 2, 4 and 12 h later.

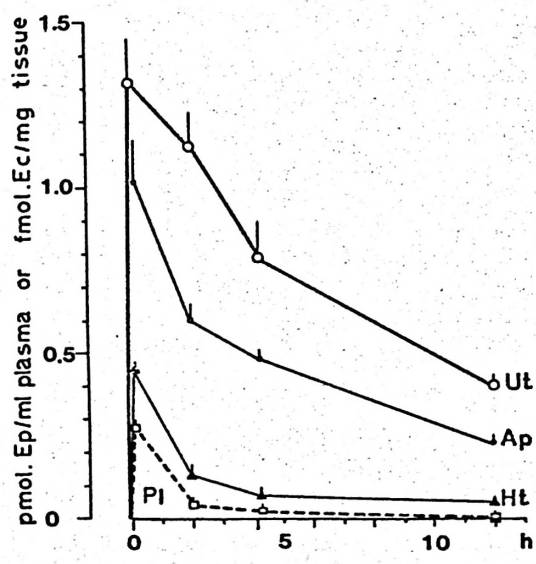

Fig. 1. *Time course of ^{3}H-Estradiol level (Ep) in plasma (Pl) expressed as pmol/ml, and cytosol uptake (Ec) of Uterus (Ut), Anterior Pituitary (Ap), and Hypothalamus (Ht) expressed as fmol/mg of wet weigh tissue.*
Determinations were effectuated at the showed time after injection of 0.5 μg of ^{3}H-estradiol by kg body weight. Each point is the mean (+ or — S.E.M.) of at least 4 separate experiments.

The plasma estradiol, measured by RIA during the rat sex cycle falls within the range of 10-80 pg or 0.03-0.22 pmol per ml of plasma (4). The plasma level of ^{3}H-E_2 10 min and 2 h after injection falls within the physiological range (from 0.29 to 0.036 pmol/ml, respectively). It declines afterwards, so that 12 h after injection, plasma radioactivity is undistinguishable from the background.

The maximal values of ^{3}H-E_2 uptake in cytosol are reached 10 min after injection (1.41, 1.02 and 0.43 fmol/mg tissue in Ut, Ap and Ht respectively) (fig. 1) and decline significantly 2 h later ($p < 0.05$ for all tissues). Twelve hours after injection the ^{3}H-E_2 level in cytosol

was 0.39 and 0.27 fmol/mg for Ut and Ap respectively, while in Ht was slighthy higher than the background.

The ^3H-E_2 uptake in Ht was higher than in Ap and Ht during the studied time. This finding is in agreement with the described Rc content for each tissue (2, 5, 6, 8, 17, 22).

The parallel profiles of plasma and cytosolic ^3H-E_2 obtained, suggests that the Rc-E_2 complex formation is closely dependent on E_2 concentration in plasma.

Fig. 2 (Ut, Ap and Ht) shows the time course for estrogen receptor level in both cytosolic and nuclear compartments after 0.5 μg of cold E_2/kg injection, and also the evolution of ^3H-E_2 uptake in both cytosol and nucleus.

Two hours after injection of cold E_2, the Rc level decreased significantly from the preinjection level of 4.2, 5.5 and 0.16 pmol/mg for Ut, Ap and Ht respectively to 2.3, 1.45 and 0.06 pmol/mg respectively ($p < 0.01$ for Ut and Ap, and $p < 0.05$ for Ht).

Four hours after injection, the replenishment of Rc is observed in all tissues; 8 h later it reaches the original level in Ap and Ht, while it is significantly higher than the basal level in Ut ($p < 0.05$). The time course for Rc in Ut, Ap and Ht after cold E_2, is a classic depletion-replenishment cycle of receptor, and is in agreement with the results of most authors, either in receptor content of tissues, or in their response to exogenous estrogens (15, 16).

The Rn level in Ap (fig. 2) increased from 1.55 fmol/mg w.w. tissue before injection to 3.3 fmol/mg w.w. tissue 2 h after cold E_2 injection ($p < 0.01$), declining afterwards to 2 fmol/mg, 12 h later. These data are in agreement with those described by other authors (1, 5, 14).

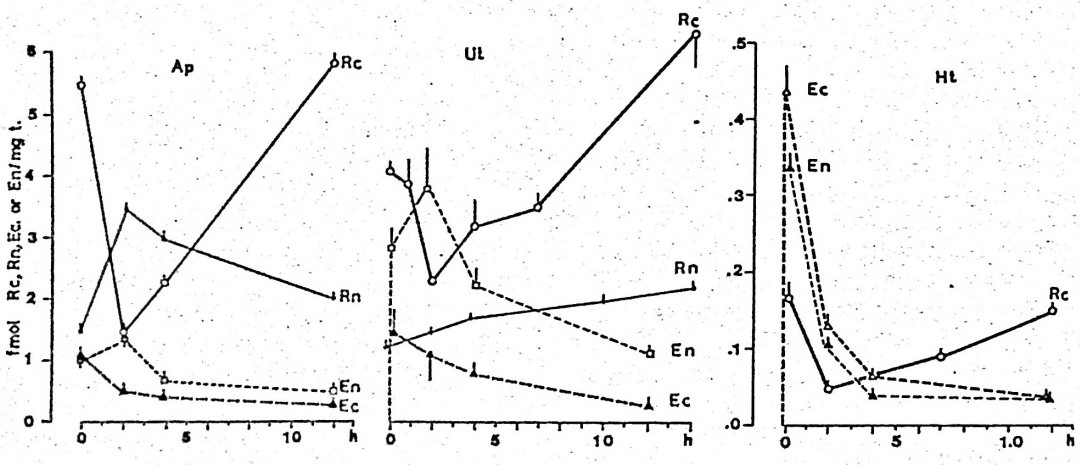

Fig. 2. *Time course of ^3H-Estradiol uptake and estrogen receptor content in Anterior Pituitary (Ap), Uterus (Ut) and Hypothalamus (Ht).*

The time course of ^3H-estradiol uptake by cytosol (Ec) and nucleus (En) of the assayed tissues, was obtained at the showed times after injection of 0.5 μg of ^3H-Estradiol/kg to castrated female rats. The results are expressed as fmol/mg w.w. tissue. Eachs point is the mean (+ or — S.E.M.) of at least 4 separate experiments. The time course of the estrogen receptor level in cytosol (Rc) and nucleus (Rn), was obtained at the showed time after injection of 0.5 μg of cold estradiol/kg of B. Wt. to castrated female rats. The receptor level was measured at the showed times after injection by means of ^3H-Estradiol exchange assays. The results are expressed as fmol of Rc or Rn/mg of w.w. tissue. Each point is the mean of at least six experiments in triplicate for Ut, or three experiments in triplicate for Ht and Ap.

The injection of 0.5 μg of cold E_2/kg provokes a slow and progressive increase of the Rn level in Ut, from 1.2 fmol/mg before injection to 2.2 fmol/mg 14 h after injection. As it can be observed in fig. 3, Rn increased during Rc depletion, but also with Rc repletion. Simultaneous Rc and Rn increase after very low E_2 doses (1 and 10 ng E_2/kg) have recently been found in this laboratory (8, 9), although they are in disagreement with the results of authors who used immature animals or larger estrogen doses (3, 6, 7, 21, 25-27, 32).

The cytosolic receptor level showed, in all assayed tissues, significant depletion-replenishment cycles. These data revealed that the 0.5 μg of cold E_2 dose affected in sufficient extent the estrogen receptor system, making unadvisable the use of larger doses of estrogens when the physiological properties of the receptor are investigated.

The time course for Rn in Ut after cold E_2 injection was slower and longer than those described by other authors (7, 19). In Ap, the Rn time course obtained was similar to that found by others (1, 14).

Uptake of ^{3}H-E_2 by Ut and Ap nuclei, reached its greatest level 2 h after injection (3.7 and 1.48 fmol/mg w.w. tissue respectively), but in Ht the greatest level is reached 10 min after injection (0.33 fmol/mg w.w. tissue) as occurs in Ht cytosol, declining afterwards.

Four hours after injection of ^{3}H-E_2, estrogen uptake in nuclei has declined significantly in all tissues; 12 h later Ap and Ut still retain significative amounts of radioactivity (0.45 and 1.2 fmol/mg w.w. tissue), while in Ht it is slighthy greater than the background.

The ^{3}H-E_2 uptake by Ap and Ut nuclei described here, are in good agreement with those described by other authors (19, 31). The data obtained for Ht (fig. 2, Ht) shows a decrease in nuclei ^{3}H-E_2 retention faster than that obtained by MCEWEN *et al.* (19) and PFAFF and KELNER (24). This finding could be due to the dose assayed here, which was 50 to 500 fold lower than that used by these authors.

The maximal ^{3}H-E_2 uptake by Ap and Ut nuclei occurred 2 h after injection, in coincidence with the maximal cytosolic receptor depletion, suggests that both processes are stoichiometrically correlated.

This observation is in disagreement with the results from other authors who showed a ^{3}H-E_2 uptake higher than the receptor level.

When using high doses of ^{3}H-E_2, the relations might not have been stoichiometric. In Ht, where the receptor content is very low, the depletion is not correlated with the rate of nuclear uptake, which may be due to an excess of the ^{3}H-E_2 dose over the hypothalamic level of estrogen receptors.

The increase of Rn in Ap was higher than the ^{3}H-E_2 uptake for its nuclei, both processes being parallel. However, the ^{3}H-E_2 uptake by the uterine nuclei was more intense than their Rn increase, and both processes were divergent.

The explanation of these findings involves necessarily the problem of the Rn origin, the mechanism of which remains still unclear (12, 13, 28). For most authors, the Rn has its origin in the estrogen receptor complex previously translocated into the nucleus and attached to the acceptor sites of chromatin (10-12). The number of chromatin acceptor sites in different estrogen responsive tissues, and their saturation by the estrogen-receptor complex (29) should be an important factor in the Rn formation process.

The similar patterns of ^{3}H-E_2 uptake obtained for Ap and Ut, and their different time courses, might well indicate that the acceptor number sites, and/or their affinity for the estrogen receptor

complex, are likely to present important differences in separate tissues. Such differences would be responsible for the nature and extent of the genomic activity affected by estrogens in their different target tissues.

Further experiments, however are necessary to confirm this hypothesis from our present work.

Resumen

Se presenta un estudio comparativo entre la captación de H^3-estradiol (^3H-E_2) y la concentración de los receptores de estradiol en tejidos diana de los estrógenos, entre las 0 y 12 horas después de la inyección de 0,5 μg/kg de ^3H-E_2 o de E_2 frío a ratas adultas ovariectomizadas.

La concentración plasmática de ^3H-E_2 entre los 10 min y las 12 h después de la inyección, se mantiene dentro del rango de los niveles plasmáticos de E_2 en ratas ciclantes. La captación total de ^3H-E_2 muestra, además, una buena correlación con el contenido de receptores en todos los tejidos diana estudiados: útero > adenohipófisis > hipotálamo > plasma. En todos los tejidos, la captación citosólica de ^3H-E_2 alcanza su máximo nivel a los 10 min de la inyección. En cada tejido se observan evoluciones paralelas entre la concentración plasmática y la captación citosólica de ^3H-E_2. La captación nuclear presenta su máximo valor a los 10 min de la inyección, disminuyendo posteriormente.

Después de la inyección de E_2 frío y en todos los tejidos, la concentración de receptores citosólicos presenta un ciclo de depleción-repleción.

En útero, la concentración de receptores nucleares aumenta progresivamente desde las 0 a las 14 horas post-inyección, si bien en adenohipófisis, su máximo nivel se alcanza a las 2 h para disminuir posteriormente.

En adenohipófisis, la captación nuclear de ^3H-E_2 y la concentración de receptores nucleares, evidencian evoluciones paralelas, coincidiendo los máximos niveles de ambos parámetros con el momento de máxima depleción de receptores citosólicos. Sin embargo, la concentración de receptores nucleares en útero aumenta más lentamente y en menor grado que la captación nuclear de ^3H-E_2, siendo ambos procesos divergentes. Estos hechos se interpretan en base a diferencias tisulares en lo que respecta a la velocidad de formación del receptor nuclear desde el complejo receptores citosólicos-E_2 previamente translocado al núcleo, y a su unión a la cromatina.

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