Dephosphorylation of oestradiol nuclear receptor *in vitro*

A hypothesis on the mechanism of action of non-steroidal anti-oestrogens

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We show that a mouse uterus nuclear phosphatase exists that is capable of inactivating nuclear oestrogen receptor complexed to oestradiol-17 β *in vitro* but is ineffective when the receptor is complexed with the two non-steroidal anti-oestrogens, nafoxidine and tamoxifen. We suggest that the long half-life of the tamoxifen–receptor complex versus the short half-life of the oestradiol–receptor complex in uterine nuclei *in vivo* is the result of the ineffectiveness of the phosphatase in dephosphorylating the anti-oestrogen–receptor complex.

In intact cells oestradiol receptor is rapidly inactivated in the nuclear compartment where it is translocated from cytoplasm by oestrogens (Gorski & Gannon, 1976).

A phosphatase inactivating oestradiol receptor *in vitro* (Auricchio *et al.*, 1980, 1981; Auricchio & Migliaccio, 1980) has been found in nuclei of oestrogen target tissues; it has been suggested that this enzyme is responsible for the inactivation of the receptor in nuclei of intact cells. In previous experiments the activity of the nuclear phosphatase was tested on cytosol receptor only. Evidence is now given that this enzyme inactivates *in vitro* the nuclear oestradiol–receptor complex too.

Furthermore two non-steroidal anti-oestrogens, tamoxifen and nafoxidine, which promote the translocation of oestradiol receptor to nuclei and cause in intact cells a much slower nuclear receptor loss than that observed with oestrogens (Clark *et al.*, 1973; Katzenellenbogen & Ferguson, 1975; Capony & Rochefort, 1975; Koseki *et al.*, 1977; Horwitz & McGuire, 1978; Jordan *et al.*, 1980) are shown to protect this receptor from inactivation by the nuclear phosphatase *in vitro*. These results offer an interpretation of the mechanism of action of these non-steroidal anti-oestrogens.

Experimental

Materials

Oestradiol-17 β and activated charcoal (Norit A) were from Sigma Chemical Co., St. Louis, MO, U.S.A. CM-52-cellulose was purchased from Whatman, Springfield Mill, Maidstone, Kent, U.K. Dex-

tran T70 was from Pharmacia Fine Chemicals, Uppsala, Sweden; gelatin was from Serva, Heidelberg, Germany. [2,4,6,7-³H]Oestradiol-17 β (sp. radioactivity 85 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks, U.K. Tamoxifen [*trans*-1-(*p*-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene; I.C.I. 46474] was from Imperial Chemical Industries, Alderley Park, Macclesfield, Cheshire, U.K. Nafoxidine hydrochloride (1-[2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl]pyrrolidine hydrochloride) was from Upjohn, Kalamazoo, MI, U.S.A.

Buffers

The following buffer solutions were used: 10 mM-Tris/HCl, pH 7.4, containing 1 mM-dithiothreitol, 1 mM-EDTA without (TED buffer) or with 0.25 M-sucrose (TED-sucrose buffer). When ZnCl₂ was added EDTA was omitted.

Tissue homogenization and fractionation

Uterus from female Swiss mice and calves was used either immediately after death or after freezing in liquid N₂ followed by thawing. Tissues were mixed with 10 vol. (mouse uterus) or 4 vol. (calf uterus) of cold TED-sucrose buffer and homogenized by a previously reported procedure (Rotondi & Auricchio, 1979). The 750 g pellet (nuclei) was obtained by centrifugation of homogenate at 2°C for 10 min and suspended in a volume of TED-buffer corresponding to half the homogenate. It was left about 30 min at 0°C before use. Cytosol was prepared either from homogenate or from 750 g

supernatant by centrifugation at 150 000 *g* for 45 min at 2°C in an L2-65 Spinco-Beckman ultracentrifuge with a Ti 50 rotor.

Preparation of cytosol and nuclear receptor–ligand complexes and inactivation experiments at 25°C

Oestradiol-17 β , tamoxifen and nafoxidine cytosol–receptor complexes were prepared by incubating calf uterus cytosol with either 12 nM-oestradiol-17 β or 100 nM-tamoxifen or 200 nM-nafoxidine for 18 h at 0°C. Portions of cytosol were then incubated at 25°C for 20 min, in the presence of either TED buffer (control) or nuclear receptor-inactivating phosphatase purified from calf uterus by a previously reported procedure (Auricchio *et al.*, 1981) in a ratio of 2:1 by vol. Immediately after incubation at 25°C the samples were cooled at 0°C and the pellet of 250 μ l of ice-cold dextran-coated charcoal suspension (1% Norit A, 0.05% dextran T 70 and 0.5% gelatine made in TED buffer) was added to 250 μ l of sample to remove the free ligand. After 5 min the samples were centrifuged, then submitted to the NaSCN exchange method.

Oestradiol-17 β –, tamoxifen– and nafoxidine–nuclear complexes were prepared in the following way; 7-day-ovariectomized mice were intraperitoneally injected with either 0.5 μ g of oestradiol, 100 μ g of tamoxifen or 200 μ g of nafoxidine and killed at the indicated times. Nuclei were prepared as described in the previous section. They were either directly utilized for the NaSCN exchange method or incubated at 25°C for 20 min and cooled at 0°C immediately after incubation, then submitted to the NaSCN exchange method.

NaSCN exchange method for the assay of cytosol and nuclear receptor

Assays were performed by a previously published procedure (Sica *et al.*, 1980). All the operations were carried out at 4°C. To 180 μ l of receptor preparations was added 20 μ l of TED buffer containing [³H]oestradiol-17 β at a final concentration of 20 nM with or without a 500-fold excess of non-radioactive hormone. After 15 min 50 μ l of TED buffer containing 2.5 M-NaSCN was added to each sample; NaSCN allows both extraction of receptor from nuclei and exchange of cold ligand bound to the receptor with [³H]oestradiol-17 β . After incubation of the sample for 16 h, 0.5 ml of cold dextran-coated charcoal suspension was added and after a further 10 min the sample was centrifuged for 10 min at 3200 *g* and the radioactivity of 250 μ l of supernatant was assayed. Specific binding activity was computed by subtracting from the binding activity of a sample with radioactive hormone the binding activity of a sample with radioactive and excess of cold oestradiol-17 β (aspecific binding).

Radioactivity assay

Samples (250 μ l) were added to 3 ml of Instagel (Packard Instrument Co., Downers Grove, IL, U.S.A.) in scintillation vials and the radioactivity was measured in a Beckman LS-7000 T counter with 45% efficiency.

Results

Evidence that nuclear phosphatase inactivates oestradiol-17 β nuclear receptor

The experiments presented in Table 1 show that receptor translocated *in vivo* to mouse uterus nuclei by oestradiol is inactivated by incubation of nuclei at 25°C. This inactivation seems to be due to the same nuclear phosphatase that is capable of inactivating cytosol receptor (Auricchio & Migliaccio, 1980; Auricchio *et al.*, 1981), since the same phosphatase inhibitors as well as the same phosphatase substrate, *p*-nitrophenyl phosphate, protect cytosol (Auricchio *et al.*, 1981) as well as nuclear receptor from the receptor-inactivating phosphatase.

Time-dependent effect of oestradiol and tamoxifen on oestradiol receptor content of nuclei

Non-steroidal anti-oestrogens as well as oestrogens promote the translocation of receptor from cytoplasm to the nuclear compartment (Clark *et al.*,

Table 1. *Prevention by phosphatase inhibitors and 4-nitrophenyl phosphate of inactivation of oestradiol mouse nuclear receptor in vitro*

Ten mice were injected with 0.5 μ g of oestradiol 1 h before being killed. Nuclei were prepared from uterus homogenate in TED-sucrose buffer and washed twice with the same buffer. They were suspended in TED buffer and incubated at 25°C for 20 min in the absence and in the presence of the indicated compounds. After incubation the samples were immediately cooled and nuclear specific binding activity was assayed using the NaSCN exchange method. The values are averages of two experiments $\pm$ s.e.m. The 100% binding activity was 0.55 nmol of specific oestrogen-binding sites/litre (104 000 $\pm$ 4200 d.p.m./ml of incubation mixture).

Compound added	Residual nuclear binding activity	
	(d.p.m./ml)	(%)
None	57 200 $\pm$ 1600	55
20 mM-NaF	92 500 $\pm$ 2500	89
5 mM-Na ₂ MoO ₄	105 000 $\pm$ 2000	101
0.5 mM-ZnCl ₂	95 600 $\pm$ 1400	92
1 mM-Na ₃ PO ₄	89 400 $\pm$ 1300	86
1 mM-sodium 4-nitrophenyl phosphate	105 000 $\pm$ 3300	101

1973; Katzenellenbogen & Ferguson, 1975; Capony & Rochefort, 1975; Koseki *et al.*, 1977; Horwitz & McGuire, 1978; Jordan *et al.*, 1980). Nevertheless receptor translocated by anti-oestrogens is slowly inactivated in nuclei of intact cells, whereas receptor translocated by oestrogens is rapidly inactivated. As a consequence of this fact the oestrogen nuclear receptor content of rat uterus is much higher 6 and 24 h after injection of tamoxifen than after injection of oestradiol (Jordan *et al.*, 1980). The opposite occurs 2 h after injection. We observe similar results using mice instead of rats (Fig. 1). The experiments presented in the following section had the purpose of verifying whether the long nuclear retention of receptor translocated by tamoxifen observed *in vivo* could be explained by a failure of the nuclear phosphatase to inactivate the anti-oestrogen-receptor complex.

Failure of the nuclear phosphatase to inactivate anti-oestrogen-receptor complex in vitro

Mice were killed 1 h after oestradiol injection and 24 h after tamoxifen or nafoxidine injections. Different times of death were selected to get a high level of uterus nuclear receptor (see Fig. 1). Uterus nuclei were prepared and incubated at 25°C. Receptor translocated by oestradiol is inactivated at 25°C as expected from the experiment presented in Table 1.

Fig. 1. Time-dependent effect of oestradiol and tamoxifen on nuclear oestrogen-receptor level

Ovariectomized mice were injected intraperitoneally (after 7 days) with 1 µg of oestradiol (●) or 100 µg of tamoxifen (▲) and killed in groups of eight at the indicated times. Nuclei were prepared from homogenate in TED-sucrose buffer, washed twice with the same buffer and suspended in TED buffer. Nuclei and cytosol prepared from nuclear supernatant were separately assayed for specific oestradiol binding activity using the NaSCN exchange method. The total (nuclear plus cytosol) specific oestrogen binding sites were 1.5 nmol/litre.

Table 2. Comparison of *in vitro* stability of anti-oestrogen- with oestrogen-nuclear and -cytosol receptor complexes

Expt. 1: Ovariectomized mice were intraperitoneally injected (after 7 days) in groups of seven with 1 µg of oestradiol 1 h before being killed, or with 100 µg of tamoxifen or 200 µg of nafoxidine 24 h before being killed. Nuclei were prepared from uterus and incubated at 25°C for 20 min. Portions of each suspension were assayed for the specific oestradiol-binding activity by the NaSCN exchange method. Expt. 2: 300 µl of suspension of nuclei prepared as in expt. 1 from mice injected with oestradiol or tamoxifen was incubated at 25°C for 20 min with 600 µl of mouse uterus cytosol prelabelled at 0°C for 18 h with 12 nM-[³H]oestradiol-17β. The specific oestradiol-binding activity of these samples was computed after separation of bound from free hormone by charcoal treatment. Expt. 3: Calf uterus cytosol samples were pre-incubated with 12 nM-oestradiol-17β, 100 nM-tamoxifen or 200 nM-nafoxidine for 18 h at 0°C. Portions (600 µl) were incubated for 20 min at 25°C with 300 µl of purified receptor-inactivating phosphatase preparation containing 4 arbitrary units (Auricchio *et al.*, 1981). Samples were then treated with dextran-coated charcoal to remove the excess of free ligand, then assayed for specific oestradiol-binding activity by the NaSCN exchange method. The 100% binding activities were 0.80, 0.89 and 1.49 nmol of specific oestrogen-binding sites/litre in expts. 1, 2 and 3 respectively.

	Residual binding activity	
	(d.p.m./ml)	(%)
Expt. 1 (nuclei from injected mice)		
With oestradiol-17β	68 000	45
With tamoxifen	149 000	99
With nafoxidine	146 000	97
Expt. 2 ([³ H]oestradiol-17β-cytosol receptor complex + nuclei from injected mice)		
With oestradiol-17β	109 000	65
With tamoxifen	112 000	67
Expt. 3 (nuclear phosphatase)		
+Oestradiol-cytosol receptor complex	167 000	60
+Tamoxifen-cytosol receptor complex	279 000	100
+Nafoxidine-cytosol receptor complex	273 000	98

In contrast receptor translocated by anti-oestrogens is stable (Table 2, expt. 1). This stability is not due to the lack of receptor-inactivating activity. In fact nuclei containing the receptor translocated by tamoxifen inactivate [^3H]oestradiol-17 β -cytosol receptor complex at 25°C to the same extent as nuclei from mice injected with oestradiol-17 β (Table 2, expt. 2).

Equal amounts of nuclear phosphatase inactivating the receptor purified from calf uterus (Auricchio *et al.*, 1981) were incubated in TED buffer for 20 min at 25°C with equal amounts of oestradiol- or anti-oestrogen-calf-uterus-cytosol-receptor complexes and receptor inactivation followed. Also in this system the hormone-receptor complex is partially inactivated (60% of residual cytosol specific oestradiol-17 β binding activity), whereas anti-oestrogen-receptor complexes are stable (Table 2, expt. 3). These results confirm that the enzyme discriminates between oestrogen and anti-oestrogen-receptor complexes.

Discussion

Evidence has recently been given that cytosol oestradiol-17 β receptor *in vitro* is inactivated by a nuclear phosphatase (Auricchio & Migliaccio, 1980; Auricchio *et al.*, 1981). This enzyme is present in oestrogen target tissue only and has extraordinary affinity for oestrogen receptor. The present results show that receptor translocated *in vivo* to the nuclear compartment by oestradiol is inactivated during incubation at 25°C of mouse uterus nuclei by the same nuclear phosphatase capable of inactivating cytosol receptor (Auricchio & Migliaccio, 1980; Auricchio *et al.*, 1981).

Furthermore there is an evident analogy between the stability of cytosol and nuclear receptor-anti-oestrogen complexes *in vitro* towards the nuclear phosphatase and the long nuclear retention of these complexes observed *in vivo* in mouse uterus and long before in MCF-7 cells (Horwitz & McGuire, 1978) and rat uterus (Jordan *et al.*, 1980).

The fact that anti-oestrogens bound to receptor appear to prevent the nuclear inactivation of receptor both *in vitro* and *in vivo* supports our thesis that the nuclear phosphatase acting on receptor *in vitro* inactivates nuclear receptor *in vivo* too.

Previous reports suggest that anti-oestrogens exert their effect by prolonging the nuclear retention of receptor and preventing cytoplasmic receptor replenishment (Clark *et al.*, 1973; Katzenellen-

bogen & Ferguson, 1975; Capony & Rochefort, 1975). Since our present results suggest that this prolonged retention is due to the failure of the nuclear phosphatase to inactivate anti-oestrogen-receptor complexes we propose that anti-oestrogens act by protecting the receptor in the nuclear compartment against dephosphorylation. This protection could also reduce cytoplasmic receptor replenishment by preventing recycling of receptor through a rephosphorylation of the inactivated receptor. The portion of cytoplasmic receptor replenishment independent of protein synthesis (Mester & Baulieu, 1975; Hsueh *et al.*, 1976) would in this way be eliminated.

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