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Changes in the Subcellular Distribution of the Rat Uterus Oestrogen Receptor as Induced by Oestradiol, Tamoxifen and ZD 182,780

A. FERREIRA MENDES, M. M. CARAMONA AND M. CELESTE LOPES

Laboratório de Farmacologia, Faculdade de Farmácia, Universidade de Coimbra, 3000 Coimbra, Portugal

Abstract

The aim of this work was to compare the subcellular distribution of the oestrogen receptor from the uteri of rats treated with vehicle alone (control group), oestradiol or one of the antio-estrogenic drugs tamoxifen and ZD 182,780. The nuclear, microsomal and cytosolic oestrogen receptor contents were evaluated by an immunoenzymatic method ("ER-EIA" kit from Abbott Laboratories) and the results in each fraction were expressed as a percentage of the total number of receptors. Parallel studies were performed to assess the uterotrophic effect of these drugs and to assess that they had reached the uterus.

In the control group, we found that the oestrogen receptor was distributed mainly between the microsomal ($29{\cdot}1 \pm 1{\cdot}3\%$) and cytosolic ($68{\cdot}1 \pm 0{\cdot}9\%$) fractions, with only a small amount located in the nucleus ($2{\cdot}8 \pm 0{\cdot}5\%$). When oestradiol was administered, the oestrogen receptor distribution was: nuclear $11{\cdot}7 \pm 2{\cdot}0$, microsomal $15{\cdot}5 \pm 1{\cdot}3$ and cytosolic $72{\cdot}8 \pm 3{\cdot}3\%$ and, in the tamoxifen group, the results were: nuclear $18{\cdot}5 \pm 1{\cdot}5$, microsomal $26{\cdot}0 \pm 3{\cdot}1$ and cytosolic $55{\cdot}5 \pm 3{\cdot}4\%$, which shows a relative shift both to the control and the oestradiol-treated groups. In the uteri of rats treated with ZD 182,780 the results were very similar to those obtained in the control group.

Our results indicate that the subcellular distribution of the oestrogen receptor varies according to the drug administered and that this receptor may not be located in a single subcellular compartment. Moreover, the nuclear uptake of the ZD 182,780-oestrogen receptor complex seems to be blocked, possibly due to impaired receptor dimerization. In the case of tamoxifen, the intracellular transport of the receptor also seems to be blocked, probably due to the nuclear retention of the receptor, thus suggesting that tamoxifen must impair the oestrogen receptor function on a step subsequent to the receptor dimerization.

The subcellular localization of the oestrogen receptor in oestrogen target tissues has long been investigated, but some aspects of this subject remain unresolved (Levin et al 1993). The initial two-step model admits a cytoplasmic localization for the unoccupied oestrogen receptor (Gorski et al 1968; Jensen et al 1968), whereas the one-step model, later postulated, admits that the oestrogen receptor resides exclusively in the nucleus, where it is activated by the hormone binding (Green et al 1984; Welshons et al 1985). Another hypothesis put forward, admits the possibility that the oestrogen receptor exists in a dynamic equilibrium between the plasma membrane, cytoplasm and nucleus (Parikh et al 1987). Moreover, the oestrogen receptor itself can no longer be considered a single and unchangeable molecule, and is now conceived as a dynamic entity that may present different features (Auricchio et al 1984; Pratt et al 1992; Levin et al 1993). Levin et al (1993) suggested a three-step model for the modulation of the genomic transcription by steroid hormones. In agreement with this model, the hormone binds to a cytoplasmic receptor/transporter for its passage to the nucleus, where it binds to and activates a nuclear oestrogen receptor. Whether the cytoplasmic receptor/transporter and the nuclear receptor correspond to one single protein suffering transformations, or to two distinct entitites, needs further investigation.

Correspondence: A. Ferreira Mendes, Laboratório de Farmacologia, Faculdade de Farmácia, Universidade de Coimbra, 3000 Coimbra, Portugal.

The triphenylethylenic anti-oestrogens and tamoxifen in particular, are widely used in the treatment of breast cancer, but their molecular mechanism of action is not yet clearly understood. Besides the oestrogen receptor and the anti-oestrogen binding sites, several other targets have been reported (Lopes et al 1991; Fanidi et al 1992; Croxtall et al 1994), including calmodulin activity (Lopes et al 1990), the Ca^{2+}-transport systems (Malva et al 1990) and molecules of cell signalling (Fanidi et al 1992; Friedman 1993; Pratt & Pollak 1993). At the oestrogen receptor level, tamoxifen is, nowadays, believed to act by interfering with portions of the receptor involved in transcription activation (Van den Koedijk et al 1994). It has been shown that the oestrogen receptor contains two transcription activation functions (Tora et al 1989), which, apparently, act in a cell- and promoter-specific fashion (Tora et al 1989; Berry et al 1990). Moreover, tamoxifen and its metabolites seem to interfere only with one of the transcription activation functions (Gronemeyer et al 1992).

ZD 182,780 is the latest of a series of compounds (the 7α-alkylamide analogues of 17β-oestradiol) considered to be pure anti-oestrogens (Wakeling & Bowler 1992). In addition, ZD 182,780 has the highest in-vivo potency as an anti-oestrogen, when compared with the other members of the series, making it a good candidate for clinical evaluation in the treatment of breast cancer (Wakeling & Bowler 1992).

Whether the anti-oestrogenic drugs interfere with the subcellular distribution of the oestrogen receptor may be

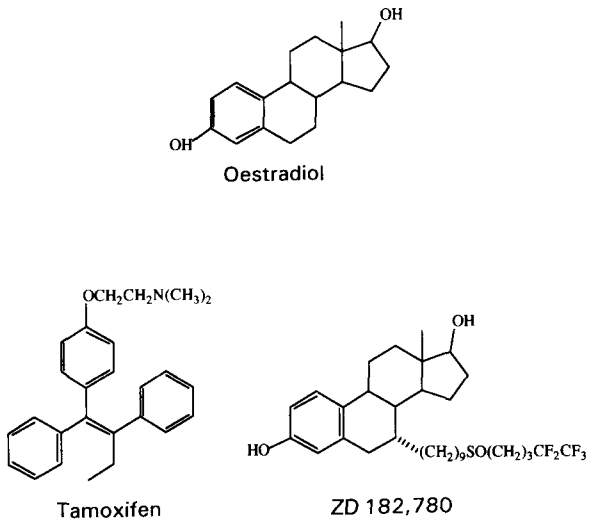

FIG. 1. Structures of 17β-oestradiol, tamoxifen and ZD 182,780.

related to the mechanism by which they interact with this receptor at the molecular level. The aim of this work was to evaluate the subcellular distribution of the oestrogen receptor in the uteri of rats treated with oestradiol, tamoxifen, ZD 182,780 (Fig. 1) or vehicle alone (control group). Parallel studies were performed to assess the uterotrophic effect of these drugs and to assess that they had reached the uterus.

Materials and Methods

Animals

Wistar female rats, 10 to 12 weeks old, with free access to food and kept at a temperature of 23 ± 2°C, with dark/light cycles of 12 h were used. All animals were ovariectomized one week before the subcutaneous administration of either vehicle alone (saline with 2% ethanol), oestradiol (220 μg kg^{-1}), tamoxifen (440 μg kg^{-1}*) or ZD 182,780 (720 μg kg^{-1}*). Eighteen hours after administration, animals were killed by decapitation and the uteri immediately excised and stripped of the adhering fat. Uteri were rinsed in ice-cold homogenization buffer, wiped and kept frozen at −80°C until processed for oestrogen receptor analysis.

Separation of the cytosolic, microsomal and nuclear fractions

Unless otherwise stated, all procedures were carried out at 4°C or on ice. Uteri were homogenized in ice-cold buffer (TEMG: 10 mM Tris-HCl, 1·5 mM EDTA, 10% glycerol and 0·1% monothioglycerol, pH 7·4) in a polytron homogenizer, set at speed 3, with 2 bursts of 5 s and 20 s on ice between them and centrifuged at 800 *g*, for 15 min. The pellet was washed twice in 1 mL TEMG buffer and the supernatants were pooled and centrifuged at 10 000 *g*, for 20 min. The supernatant was then ultracentrifuged at 180 000 *g*, for 1 h. The pellet (microsomal fraction) was resuspended in ice-cold buffer and the supernatant constituted the cytosolic fraction.

The pellet from the 800-*g* centrifugations (crude nuclear pellet) was treated as described by Kendall & Rose (1992) and centrifuged at 105 000 *g*, for 30 min. The supernatant (nuclear extract), the cytosol and the microsomal fraction were kept at −80°C and used within a month for the oestrogen receptor determination.

Oestrogen receptor determination

An immunoenzymatic method, the ER-EIA kit obtained from Abbott Laboratories, North Chicago, IL, was used for the oestrogen receptor determinations following the manufacturer's instructions. The protein concentration of each sample was determined using the Bio-Rad Protein Assay kit from Bio-Rad Laboratories GmbH, München, West Germany. Whenever needed, protein content was corrected to 1–2 mg mL^{-1} by dilution with the homogenization buffer, as required for proper use of the ER-EIA kit.

DNA determination

DNA was measured by the method of Burton (1956), using an aliquot of the crude nuclear pellet.

Reagents

Oestradiol was purchased from Sigma (St Louis, MO) and tamoxifen from Amersham (Bucks, UK). ZD 182,780 was kindly supplied by Zeneca Produtos Biociência, Portugal. All other reagents used were analytical grade and purchased from Sigma or Merck.

*Equimolar doses.

Results and Discussion

Since the DNA content is constant from cell to cell and reflects the number of cells in a given tissue or organ, we measured the DNA content of the uteri from rats treated with oestradiol, tamoxifen and ZD 182,780 to measure their proliferative or antiproliferative effect relative to the control group. Our results indicate that tamoxifen acted, in this system, as a partial agonist. Tamoxifen induced an increase in the DNA content (54·3 ± 4·0 μg/uterus), relative to that found in the control animals (45·9 ± 1·5 μg/uterus) (Table 1). This increase was less pronounced than that produced by treatment with oestradiol (77·0 ± 4·0 μg/uterus) (Table 1). Upon ZD 182,780 administration, the DNA content (41·3 ± 2·1 μg/uterus) was identical to that of the control group (45·9 ± 1·5 μg/uterus) (Table 1), indicating that ZD 182,780 had no agonistic properties. Furthermore, in some experiments in which ZD 182,780 was administered in association with oestradiol (doses as referred above for single administration), DNA content was smaller than that obtained after oestradiol alone, but higher than when only ZD 182,780 was given (data not shown). These results indicate that ZD 182,780 acts as a full antagonist in this system, thus agreeing with the proposal that it is a pure anti-oestrogen (Wakeling & Bowler 1992), although slight oestrogenic actions have also been noticed by some investigators (Lyttle et al 1992).

To evaluate the subcellular distribution of the oestrogen receptor in the uteri of control and treated rats, we analysed, besides the nuclear and cytosolic fractions, the microsomal fraction, since it has been reported that the endoplasmic reticulum also contains an appreciable amount of oestrogen-binding sites (Muldoon et al 1988; Lopes et al 1991). For this purpose, we used monoclonal antibodies instead of the classical radioligand binding assay. That method allows

Table 1. DNA content of the uteri from rats treated with vehicle alone (control), oestradiol, tamoxifen or ZD 182,780.

	DNA content (μg DNA/uterus)
Control	45·9 ± 1·5
Oestradiol	77·0 ± 4·0*
Tamoxifen	54·3 ± 4·0*†
ZD 182,787	41·3 ± 2·1*†

Values given are means ± s.e.m. (n = 5). Results were analysed with one-way analysis of variance. *$P < 0.05$ compared with control, †$P < 0.001$ compared with oestradiol.

the evaluation of the whole receptor population, whether occupied or not (Jordan et al 1986), thus reflecting more accurately the influence of different ligands on the subcellular distribution of the oestrogen receptor. We expressed the results in the nuclear, cytosolic and microsomal fractions as a percentage of the total number of receptors (Table 2). In this way, the oestrogen receptor distribution becomes more clear than when the results are expressed as a concentration (fmol receptor (mg protein)$^{-1}$ for the cytosolic and microsomal fractions and fmol receptor (mg DNA)$^{-1}$ for the nuclear fraction).

Our results indicate that the subcellular distribution of the oestrogen receptor varies according to the drug administered. In the control group, we found that the oestrogen receptor was distributed mainly between the microsomal (29·1 ± 1·3%) and cytosolic fractions (68·1 ± 0·9%), with only a small amount located in the nucleus (2·8 ± 0·5%) (Table 2). When oestradiol was administered, the oestrogen receptor location changed dramatically, relatively to the control group, with almost half of the microsomal receptors disappearing and a simultaneous increase in the nuclear percentage, while the increase in the cytosolic percentage was not significant (nuclear 11·7 ± 2·0, microsomal 15·5 ± 1·3 and cytosolic 72·8 ± 3·3%) (Table 2).

In the uteri of rats treated with tamoxifen, we did not observe the reduction in the microsomal oestrogen receptor content apparent with oestradiol relative to the control group, but there seems to have occurred a transfer to and retention in the nucleus of cytosolic receptors (nuclear 18·5 ± 1·5, microsomal 26·0 ± 3·1, cytosolic 55·5 ± 3·4%) (Table 2). Interestingly, the percentage of nuclear receptors was greater in the tamoxifen-treated group than in the oestradiol group, despite the much smaller affinity of tamoxifen for the oestrogen receptor (Wakeling & Bowler 1988).

Table 2. Subcellular distribution of the oestrogen receptor in uterine homogenates from rats treated with oestradiol, tamoxifen, ZD 182,780 or vehicle alone (control). The oestrogen receptor content of each fraction is expressed as a percentage of the total number of receptors.

	Oestrogen receptors (%)		
	Nuclear	Microsomal	Cytosolic
Control	2·8 ± 0·5	29·1 ± 1·3	68·1 ± 0·9
Oestradiol	11·7 ± 2·0*	15·5 ± 1·3*	72·8 ± 3·3
Tamoxifen	18·5 ± 1·5*	26·0 ± 3·1†	55·5 ± 3·4†
ZD 182,780	3·1 ± 0·6†	30·2 ± 1·8†	66·7 ± 2·2

Values represent mean ± s.e.m (n = 5). All samples were assayed in duplicate. Results were analysed with one-way analysis of variance. *$P < 0.05$ compared with control; †$P < 0.05$ compared with oestradiol.

The administration of ZD 182,780 induced a distribution of the oestrogen receptor (nuclear 3·1 ± 0·6, microsomal 30·2 ± 1·8, cytosolic 66·7 ± 2·2%) that was identical to that obtained in the control group (Table 2). ZD 182,780 has a relative binding affinity for the oestrogen receptor (89% relative to oestradiol) (Wakeling & Bowler 1992) that is significantly greater than that of tamoxifen (2·5%) (Wakeling & Bowler 1988). Thus, one would expect the nuclear retention of the ZD 182,780-oestrogen receptor complex to be even greater than that obtained upon tamoxifen administration and, in this group, it should be inferior to the nuclear oestrogen receptor content found in the oestradiol-treated group, but this was not observed. Accordingly, the one-step model seems insufficient to explain these results.

The different percentages of microsomal receptors found in the uteri of rats treated with the various drugs also can not be explained by the one-step model. The greater proportion of cytosolic receptors in the oestradiol-treated group, relative to the control (Table 2), is probably due to the solubilization of the microsomal receptors induced by oestradiol as suggested by Muldoon et al (1988). Upon tamoxifen or ZD 182,780 administration, the microsomal oestrogen receptor content is, in both cases, identical to that obtained in the control group (Table 2), although in the nuclear and cytosolic fractions the results are quite different. Thus, both anti-oestrogens impair the participation of the microsomal system in the oestrogen receptor function. It appears that tamoxifen causes retention of the oestrogen receptor in the nucleus, without interfering with the microsomal receptors, or, otherwise, that the nuclear retention prevents the solubilization and intracellular transport of the oestrogen receptor that occurs in the presence of oestradiol.

Upon ZD 182,780 administration, the intracellular transport of the receptor also seems to be blocked, but, contrary to tamoxifen, the receptor is retained in the cytosol and microsomal fraction (Table 2). Which type of alteration does it induce preventing the intracellular transport of the receptor, is not clarified by our results, which, in turn, favour the hypothesis of an inhibition of receptor dimerization, suggested both for ZD 182,780 (Parker 1993) and for its analogue ZD 164,384 (Fawell et al 1990), as a possible mechanism of action. The length of the 7α side chain of ZD 182,780 and its analogues can sterically interfere with the dimerization domain, preventing receptor transformation (Parker 1993) and, hence the nuclear uptake of the oestrogen receptor and its movement within the cell; our results appear to be in agreement with this interpretation. Consequently, the oestrogen receptor-DNA binding in-vivo would be inhibited, explaining the pure anti-oestrogenic properties reported for these drugs (Wakeling & Bowler 1992). Tamoxifen and the other triphenylethylene derivatives, lacking such a long side chain, may not interfere with the dimerization domain. Instead they may alter the conformation/structure of the oestrogen receptor in such a way that the transcription activation, located in the hormone binding domain of the oestrogen reeptor, can not interact adequately with the oestrogen responsive elements in

DNA. The other transcription activation fuction of the oestrogen receptor is not affected by tamoxifen binding to the oestrogen receptor. Thus, the agonistic or antagonistic of the triphenylethylenes would, ultimately, depend on the relative importance of each one of the two transcription activation functions in each cell type (Gronemeyer et al 1992). Our results point to the possibility that the transformation and nuclear uptake of the anti-oestrogen-oestrogen-receptor-complex do not differ from those of the oestradiol-oestrogen receptor complex, but the intracellular transport of the receptor seems to be blocked, probably due to its nuclear retention, thus further supporting the hypothesis that the triphenylethylene anti-oestrogens must impair the oestrogen-receptor function on a step subsequent to the receptor dimerization.

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