The effect of a single dose of oestradiol on tamoxifen-induced uterine hyperaemia and growth in the rat

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- 1 The effect of oestradiol on tamoxifen – (single doses of each agent) induced responses in the uterus of the mature ovariectomized rat was investigated. The parameters examined were uterine blood flow (measured by the microsphere technique), weight and oestrogen receptor concentrations.
- 2 Initially the addition of oestradiol to the tamoxifen regimen produced a reduction in uterine blood flow and dry weight which was reflected by a reduction in nuclear oestrogen receptor. However, as the tamoxifen response became more established from 48 h onwards the addition of oestradiol produced an additive effect. These changes were not mirrored by increases in nuclear oestrogen receptor.
- 3 The results suggest that tamoxifen and oestradiol may, in part, act via different mechanisms; the role of possible mediatory systems such as histamine release and eosinophil migration is discussed. Heterogeneous control systems may also be involved in the early and late uterine response.

Introduction

Tamoxifen, although generally classified as a non-steroidal antioestrogen, can, under certain conditions, act as a partial agonist. This dualism depends upon the target organ and species investigated; endocrine status may also be relevant in breast cancer patients. In the rat, tamoxifen behaves as a partial agonist (Harper & Walpole, 1967; Emmens, 1970; Jordan *et al.*, 1978). It is possible that tamoxifen exerts its effect via the oestrogen receptor system as it has been shown that the triphenylethylene antioestrogens can compete and bind to oestrogen receptor sites (Sutherland & Murphy, 1982).

After formation of the oestrogen receptor/ligand complex, a process known as transformation occurs which increases the affinity of the complex for the chromatin. Recent evidence suggests that the ligand associated with the receptor is important with respect to conformational changes of the receptor molecules (Giambiagi & Pasqualini, 1988); the end result being the specific genomic responses associated with hormone stimulation.

A previous study in this laboratory has shown that a single dose of tamoxifen has a persistent effect on uterine blood flow and weight (Marshall & Senior, 1987). As a result of this work, we suggested that the classical genomic oestrogen receptor interaction may not fully account for the uterotrophic events seen after tamoxifen administration. In the current work, oestradiol was given at various times after single dose tamoxifen administration in order

to investigate the possibility of compromising or altering the established tamoxifen uterotrophic response, thus further elucidating the relationship between the uterine response and oestrogen receptor dynamics.

Methods

Mature virgin female rats of a CD-derived Sprague-Dawley random-bred strain from the animal house, University of Bradford, were used throughout and were housed in light (07 h 00 min–19 h 00 min) and temperature (18°C) controlled rooms. Food and water were available *ad libitum*. All the animals used in this study were bilaterally ovariectomized at least 21 days before further experimentation and were randomly assigned to groups.

Measurement of blood flow

The microsphere technique (previously described by Phaily & Senior, 1978) was used to determine blood flow in the rat anaesthetized with sodium pentobarbitone (60 mg kg⁻¹). This dose was selected to ensure anaesthetic control throughout the experimental procedure and minimal cardiovascular changes. The microspheres used were 15 µm in diameter (NEN-Trac, New England Nuclear, Boston, MA, U.S.A.), uniformly labelled with ⁴⁶Sc and suspended in 10% (w/v) dextran containing 0.1% (w/v) Tween 80.

Uterine blood flows were calculated using wet weight of tissue. In all cases blood flow between the adrenals and kidneys was balanced, indicating a uniform distribution of microspheres. Cardiac output was also unaffected by the treatments. When dry weights are quoted the tissue was dried until the weight remained constant.

Measurement of cytoplasmic and nuclear oestrogen receptors by [³H]-oestradiol exchange

Homogenization, cytosol and nuclear pellet preparation and the determination of oestrogen receptors therein was carried out by the method of Marshall & Senior (1987). Briefly, animals were killed by cervical dislocation and the uteri were rapidly removed and homogenized in PE buffer (10 mM phosphate buffer, pH 7.4, containing 1.5 mM EDTA and 2 mM mercaptoethanol). All procedures were performed at 4°C unless otherwise stated. Homogenates were centrifuged at 100,000 *g* for 1 h. Aliquots of cytosol were incubated at 30°C for 1 h with 1–20 nM of [³H]-oestradiol ([2, 4, 6, 7-³H]-oestradiol, 84–104 Ci mmol⁻¹) (Radiochemical Centre, Amersham, Bucks) plus or minus 100 fold excess of oestradiol. Separation of bound from free ligand was achieved by use of dextran-coated charcoal (0.5% dextran (BDH, Poole), 5% Norit A acid washed charcoal w/v in phosphate buffer). The radioactivity in the supernatant was determined by liquid scintillation counting. The assay of nuclear oestrogen receptors involved the preparation of a low speed nuclear pellet (prepared by centrifugation at 800 *g* of homogenate). Aliquots of the nuclear suspension were treated in the same way as in the cytosolic assay. Separation was achieved with a 60% hydroxylapatite suspension in phosphate buffer. After ethanolic extraction of the pellet, radioactivity was determined by liquid scintillation counting (44% efficiency).

Drug administration

Oestradiol (oestra-1,3,5-(10)-triene-3,17 β -diol; BDH, Poole, UK) was dissolved in a 10% (v/v) solution of propylene glycol and was administered intravenously (i.v.) at a dose level of 0.5 μ g kg⁻¹ into the tail vein of the conscious rat 3 h before measurements were made. This dose of oestradiol was chosen as it gives maximal uterine blood flow 3 h after administration and is the minimum dose to produce this effect. Tamoxifen (ICI, Macclesfield, Cheshire) was dissolved in ethanol and dispersed in arachis oil before subcutaneous (s.c.) injection at a dose level of 1 mg kg⁻¹. The solvents used did not have any effect on the parameters measured and these results have been grouped as controls.

Statistical analysis

The results (expressed as mean $\pm$ s.e.mean) were compared by analysis of variance and Student's *t* test (two tailed) (Snedecor & Cochran, 1979).

Results

The exchange conditions employed were found to be optimal with respect to maximal exchange with minimal loss of binding sites.

In this work the term cytoplasmic receptor is used: this refers to the receptor form that separates into the cytoplasmic cell fraction after homogenization. It represents the unoccupied inactive form of the receptor thought to be located in the nucleus *in vivo*. The nuclear or activated form of the receptor remains in the nuclear compartment after fractionation (Jordan, 1984). Although receptor localization is important, in terms of this study the receptor measurements made are used in a qualitative manner to assess the effect of drug treatments on the receptor population as a whole, rather than as an attempt to compartmentalise the oestrogen receptor.

Effect of oestradiol on tamoxifen-stimulated blood flow (Table 1)

When oestradiol was included in the treatment regimen 24 h after tamoxifen administration and uterine blood flow was measured 3 h later, the presence of oestradiol caused significantly reduced blood flow when compared to the result with tamoxifen alone at 27 h. However this reduction was no longer apparent at 48 h after the tamoxifen treatment (oestradiol was given at 45 h); 72 h after tamoxifen administration the addition of oestradiol had caused significant elevation in uterine blood flow.

Blood flow to the adrenals, kidneys, spleen and stomach was not significantly altered by the treatments, except for splenic flow which showed an increase ($P < 0.05$) 72 h after tamoxifen injection.

Effect of oestradiol on tamoxifen-stimulated uterine weight and water content (Table 1)

In terms of uterine wet weight oestradiol caused the most significant alteration in this parameter when administered 45 h after the tamoxifen dose (measurement made 3 h after the oestradiol injection), when the addition of oestradiol caused a further elevation of wet weight to a uterus already enlarged by tamoxifen. This increase was due mainly to water imbibition in the uterus. Similarly, at this time (48 h) the inclusion of oestradiol also enhanced tamoxifen-stimulated uterine dry weight. This dry weight increase was still evident 72 h after tamoxifen administration.

Table 1 The effect of oestradiol on tamoxifen (1 mg kg^{-1} s.c., single dose)-stimulated uterine blood flow and weight in rats: measurements were made at 27 h, 48 h and 72 h after administration of tamoxifen; where the combination was used oestradiol ($0.5 \text{ } \mu\text{g kg}^{-1}$, i.v.) was injected 3 h before blood flow determinations

Pretreatment	No. of rats	Body weight (g)	Cardiac output (ml min^{-1})	Uterine blood flow		Uterine weight		Water content of uterus (mg)
				ml min^{-1}	$\text{ml min}^{-1} 100 \text{ g}^{-1}$	Wet	Dry	
None (Control)	9	304 ± 7	93 ± 6	0.065 ± 0.01	66 ± 12	107 ± 8	23 ± 3	85
Oestradiol 3 h	8	321 ± 7	104 ± 11	0.94 ± 0.10	735 ± 66	128 ± 7	35 ± 2	93
Tamoxifen 27 h	6	312 ± 14	106 ± 12	0.88 ± 0.20	649 ± 117	129 ± 9	29 ± 2	100
Tamoxifen + oestradiol (24 h + 3 h)	7	314 ± 10	82 ± 17	$0.53 \pm 0.11^*$	$376 \pm 66^{**\dagger}$	139 ± 6	$26 \pm 2^{**}$	113
Tamoxifen 48 h	6	338 ± 15	99 ± 8	0.80 ± 0.19	489 ± 123	$167 \pm 8^{**}$	$28 \pm 1^*$	139
Tamoxifen + oestradiol (45 + 3 h)	6	345 ± 12	88 ± 4	1.23 ± 0.42	633 ± 181	$190 \pm 15^{***}$	$37 \pm 4^\dagger$	153
Tamoxifen 72 h	6	360 ± 11	124 ± 12	1.58 ± 0.09	$988 \pm 56^*$	$160 \pm 7^{**}$	34 ± 1	126
Tamoxifen + oestradiol (69 + 3 h)	6	346 ± 9	98 ± 9	$2.13 \pm 0.23^{***\dagger}$	$1328 \pm 147^{**\dagger}$	$160 \pm 5^{**}$	38 ± 2	121

Values are mean $\pm$ s.e.mean; * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0001$ values are significantly different from those of oestradiol alone group. $^\dagger P < 0.05$ values are significantly different from those of the equivalent tamoxifen alone group.

Effect of oestradiol in the presence of tamoxifen on oestrogen receptor levels (Figure 1)

Three hours after treatment with oestradiol alone, cytosolic receptor concentration had decreased when compared to control values with a concomitant increase in nuclear receptor concentration. When tamoxifen was used alone, cytosolic receptor concentration was lowest at 27 h but increased with time after injection, whilst nuclear receptor levels were highest at 27 h after injection and were significantly lower at 48 h and 72 h.

Figure 1 The effect of single injections of oestradiol ($0.5 \mu\text{g kg}^{-1}$, i.v. administered 3 h before measurements were made) and tamoxifen (1 mg kg^{-1} , s.c. single dose) used singly and together on cytosolic (a) and nuclear (b) oestrogen receptor distribution in mature ovariectomized rats. In (a) and (b) untreated control, vertically lined column; oestradiol, solid column; tamoxifen, open column; oestradiol plus tamoxifen, hatched column. The results are shown at various times after tamoxifen injection. Each column represents mean of 3–6 determinations, with 6 uteri per determination; vertical bars show s.e.mean. $***P < 0.005$, $***P < 0.001$, values are significantly different from those of oestradiol alone group. $\dagger P < 0.05$, values are significantly different from those of equivalent tamoxifen alone group.

When oestradiol was included in the tamoxifen regimen it had the most marked effect when given 24 h after tamoxifen. When the measurements were made 27 h after tamoxifen administration, oestradiol caused a significant reduction in nuclear receptor content compared to treatment with tamoxifen alone; at the same time there was also a small increase in the concentration of receptor in the cytosolic compartment.

Discussion

When oestradiol is used alone it causes a shift in oestrogen receptor from the cytosolic to the nuclear compartment. A similar, although more marked movement of receptor is seen when tamoxifen is used alone at 27 h. As the time after tamoxifen administration increases, nuclear receptor concentration begins to decline but the concentration of cytosolic receptor remains depressed, compared to untreated control values. This pronounced depletion of cytosolic receptor has been well documented (Katzenellenbogen *et al.*, 1983; Kirchoff *et al.*, 1983) and may be attributable to resynthesis/recycling abnormalities with respect to the antioestrogen-receptor complex. The presence of the so-called anti-oestrogen binding site may also be relevant in this context. As Sudo *et al.* (1983) have suggested, these high affinity and widely distributed sites may influence the action of antioestrogens in a passive manner by altering their biological potency and pharmacokinetics.

In the earlier stages of the tamoxifen response (a single 1 mg kg^{-1} dose of tamoxifen exerts a uterotropic effect for up to six weeks, Marshall & Senior, 1987) the addition of oestradiol to the regimen produces a reduction in uterine blood flow and dry weight. In terms of oestrogen receptor dynamics, this decline is reflected by a decrease in nuclear oestrogen levels. Therefore, in the presence of oestradiol it would seem that tamoxifen at this stage of the response is assuming the role of an antagonist. It may also be feasible that when oestradiol is included in the regimen, tamoxifen is forced to compete for oestrogen receptor sites and some of its binding capacity is switched to another site, possibly the antioestrogen/ H_3 site described by Brandes & Bogdanovic (1986), so in effect the stimulatory effects of oestradiol are counterbalanced by a change in emphasis in tamoxifen binding.

Later in the tamoxifen response at 48 h, the effect of oestradiol on tamoxifen is manifested as an increase in both uterine wet weight (this increase is accompanied by increased uterine oedema) and dry weight. However, these differences do not appear to

be reflected in changes in oestrogen receptor levels. By 72 h after tamoxifen administration, oestradiol causes a small increase in the dry weight response. However, this additive effect is most marked in terms of the dramatic increase seen in uterine blood flow at this time, although again these changes are apparently independent of alterations in oestrogen receptor levels.

This additive effect may be attributed to the two agents acting via different mechanisms, for example the effect of oestradiol may involve histamine synthesis and release. Results from this laboratory suggest that histamine is involved in the early (3 h) response to oestradiol but that it does not appear to play a major role in the uterine response to tamoxifen (Marshall & Senior, 1986). Tamoxifen may be more dependent on, for example, the eosinophil-mediated mechanism proposed by the Tchernitchin group (Galand *et al.*, 1985; Tchernitchin, 1979; Tchernitchin, 1983) as they have shown that nafoxidine, also a triphenylethylene derivative, can induce eosinophil migration (Galand *et al.*, 1984). The reversal of the combined blood flow response at 27 h and 72 h may also indicate a different mediatory system.

The results of this work would seem to indicate

that changes in the uterotrophic parameters examined in this study do not always correspond with alterations in oestrogen receptor levels. It is difficult to correlate exactly the uterine response with oestrogen receptor dynamics. Even though receptor concentrations are measurable, it is not possible to assess the quality of the binding taking place. A study by Giambiagi & Pasqualini (1988) has shown that oestradiol, tamoxifen and its major metabolite 4-hydroxytamoxifen induce different conformational modifications of the oestrogen receptor molecule and the way it interacts with the genome. The changes in direction seen when oestradiol was added at the various stages of the tamoxifen response may be suggestive of different regulatory systems coming into play with respect to the early and later uterotrophic events. The oestrogen receptors present on the endothelial cell membrane in uterine arteries (Pietras & Szego, 1977) may be of importance in this respect as postulated by Arvidson & Terenius (1982).

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