Br. J. Pharmac (1987), **92**, 429–435

A study on the effect of a single dose of tamoxifen on uterine hyperaemia and growth in the rat

K. Marshall & J. Senior

Postgraduate School of Studies in Pharmacology, University of Bradford, Bradford, West Yorkshire, BD7 1DP

1 The effect of a single subcutaneous dose of tamoxifen on the rat uterotrophic response was investigated.

2 The parameters examined were uterine blood flow (measured by the microsphere technique), uterine wet and dry weights and the concentrations of cytosolic and nuclear oestrogen receptors.

3 Tamoxifen or its metabolites proved to be capable of eliciting a uterotrophic response of 35–42 days duration. The changes seen in uterine blood flow and weight are discussed in relation to oestrogen receptor distribution.

Introduction

There has been considerable interest in understanding the mechanism of action of the triphenylethylene anti-oestrogens because of their ability to antagonize many of the actions of oestrogen. In particular, these compounds have been shown to suppress oestrogen stimulated uterine growth in a variety of animal species, to cause regression of hormone-dependent mammary tumours and to alter gonadotrophin secretion (Jordan, 1976). However, the compounds are unusual in that they display a diversity of actions ranging from oestrogen antagonism to partial agonism in a manner that depends upon species and target tissue. In the rat tamoxifen is a partial agonist (Harper & Walpole, 1967; Emmens, 1970; Jordan *et al.*, 1978). Since tamoxifen is capable of binding to the cytoplasmic oestrogen receptor (Rochefort *et al.*, 1972; Clark *et al.*, 1973; Katzenellenbogen & Ferguson, 1975) it has been suggested that the oestrogenicity of tamoxifen is related to this property (Koseki *et al.*, 1977).

In this work the interaction of tamoxifen with the classical oestrogen receptor system was studied in relation to uterine events in the mature ovariectomised rat.

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 were bilaterally ovariectomised at least 21 days before further experimentation and were randomly assigned to groups.

Measurement of blood flow

Blood flow was measured in rats, anaesthetized with sodium pentobarbitone (60 mg kg^{-1}), by the microsphere technique described in previous work (Phaily & Senior, 1978). In the present experiments the microspheres had a mean diameter of 15 μm (NEN-Trac, New England Nuclear, Boston, MA, U.S.A.) and were uniformly labelled with ^{46}Sc and suspended in 10% (w/v) dextran containing 0.01% (w/v) Tween 80. Blood flows were calculated using wet weight of tissues. When dry weights are quoted the tissues were dried until the weight remained constant.

Drug administration

Tamoxifen (I.C.I., Macclesfield, Cheshire) was dissolved in absolute alcohol and dispersed in arachis oil before subcutaneous (s.c.) injection. The vehicle used did not have any effect on the parameters measured and these results have been grouped as controls (control measurements are representative from a large control group which did not vary significantly over a period of 2 years).

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

Animals were killed at the indicated times by cervical

dislocation. Uteri were rapidly removed, stripped of extraneous tissue, blotted, weighed and placed in chilled (4°C) P.E. buffer (10 mM phosphate buffer, pH 7.4 containing 1.5 mM EDTA and 2 mM mercaptoethanol). Tissue concentration was approximately 1 uterus 0.5 ml^{-1}. All subsequent procedures were performed at 4°C unless otherwise stated. The assay was performed in triplicate.

The uteri were finely minced, then homogenized (Polytron, setting 5) for 2 × 10 bursts with intermittent cooling. The homogenate was centrifuged at 800 *g* (MSE Magnum) for 20 min to obtain the supernatant (cytosol) and nuclear fraction.

Total cytosolic receptor sites were estimated essentially according to the procedure of Katzenellenbogen *et al.* (1973). The low speed supernatant was centrifuged at 100,000 *g* (Beckman Model L5-40) for 1 h. One hundred μl of cytosol was added to assay tubes containing varying concentrations (1–20 nM) of [^{3}H]-oestradiol (2,4,6,7-[^{3}H]-oestradiol, 84–104 Ci $mmol^{-1}$) (Radiochemical Centre, Amersham, Bucks.) plus or minus 100 fold excess of oestradiol (oestra-1,3,5(10)-triene-3,17β-diol) (B.D.H., Poole) to estimate non-specific binding. The tubes were vortexed and incubated at 30°C for 1 h. Separation of bound from free ligand was achieved by the addition of 100 μl dextran-coated charcoal (0.5% dextran, (B.D.H., Poole), 5% Norit A acid washed charcoal wt/vol in phosphate buffer). After a 15 min incubation, tubes were centrifuged (1 min in Eppendorf bench centrifuge) and 100 μl of supernatant was added to 4 ml of Scintran Cocktail T (B.D.H., Poole).

Nuclear receptor sites were estimated essentially according to the procedure of Clark & Peck (1979). The low speed nuclear pellet was washed by resuspension (approximately 1 ml to 50 mg uterus) and centrifugation (800 *g* for 10 min) and the final pellet was resuspended (same ratio as above). Two hundred μl of the nuclear suspension was pipetted into assay tubes as used in cytosolic receptor assay. Tubes were incubated at 30°C for 1 h. Separation was achieved by the addition of 200 μl of 60% hydroxylapatite suspension in P.E. buffer. After 15 min the tubes were centrifuged (5000 *g* for 5 min), and resuspended in P.E. buffer 3 times. The washed pellet then underwent ethanolic extraction (incubated at 30°C for 30 min) after which the tubes were centrifuged (800 *g*, 10 min) and 200 μl of the supernatant were added to 4 ml of Scintran Cocktail T for counting.

Samples were counted in a Packard-Tricarb Model 460C liquid scintillation spectrometer at 41% efficiency. Automatic external standardization was employed to determine the degree of quenching in each sample. Specific binding was calculated as the difference between total and non-specific binding. The quantities of cytoplasmic and nuclear receptor sites were estimated by Scatchard analysis (Scatchard, 1949). Cytosolic receptor concentrations are expressed per mg protein, determined using the method of Lowry *et al.* (1951) and bovine serum albumin as the standard. Nuclear receptor concentrations are expressed per mg DNA, determined using the method of Burton (1956) with calf thymus DNA as a standard.

Statistical analysis

The results (expressed as mean ± s.e.mean) were compared by use of 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.

Effect of tamoxifen on blood flow (see Table 1)

The most striking feature of the effect of tamoxifen on uterine blood flow was the duration of the response – it took 49 days for blood flow to return to near control values (as opposed to 72 h after a single injection of oestradiol – Marshall & Senior, unpublished observation). Two hundred and sixty four hours (11 days) after the injection of tamoxifen, uterine blood flow reached a maximum which was approximately 350 ml min^{-1} 100 g^{-1} greater than that seen with oestradiol (uterine blood flow peaks 3 h after a single 0.5 μg kg^{-1} i.v. dose of oestradiol at 735 ± 66 ml min^{-1} 100 g^{-1} – Marshall & Senior, unpublished observation). Blood flow to the adrenals, kidneys and stomach was not significantly altered by tamoxifen treatment. However, blood flow to the spleen was increased 72 h, 672 h and 1176 h ($P < 0.05$) after injection. In all cases blood flow between the adrenals and kidneys was balanced indicating a uniform distribution of microspheres. Cardiac output was unaffected by the treatments.

Effect of tamoxifen on uterine weight and water content (see Table 1)

Tamoxifen increased uterine wet weight significantly at 30 h, 48 h, 72 h and 96 h after injection when compared with the untreated control group. Uterine dry weight was significantly elevated for each successive measurement between 27 h and 96 h after injection, with futher increases seen at 216 h (9 days), 264 h (11 days), 336 h (14 days) and 1008 h (42 days). Both uterine wet and dry weight decreased significantly, compared with control group, at 840 h (35 days) after injection. Uterine water content reached its maximum 48 h after tamoxifen treatment, fluctuating around

Table 1 The effect of a single subcutaneous injection of tamoxifen (1 mg kg^{-1}) on uterine blood flow and weight in rats

Time after injection (h)	*No. of rats*	*Body weight* (g)	*Cardiac output* (ml min^{-1})	*Uterine blood flow* (ml min^{-1})	*Uterine blood flow* (ml min^{-1} 100 g^{-1})	*Uterine weight* (mg) *Wet*	*Uterine weight* (mg) *Dry*	*Water content of uterus* (mg)
0 (Control)	6	342 ± 11	93 ± 13	0.048 ± 0.008	44 ± 6	108 ± 8	22 ± 1	85
24	7	331 ± 6	113 ± 11	0.52 ± 0.11**	406 ± 87**	130 ± 8	26 ± 2	104
27	6	312 ± 14	106 ± 12	0.88 ± 0.20**	689 ± 117**	129 ± 9	29 ± 2*	100
30	6	320 ± 12	86 ± 15	1.10 ± 0.19***	838 ± 152***	132 ± 4*	29 ± 2*	104
48	6	338 ± 15	99 ± 8	0.80 ± 0.19**	489 ± 123**	167 ± 8***	28 ± 1**	139
72	6	360 ± 11	124 ± 12	1.58 ± 0.09***	988 ± 56***	160 ± 7**	34 ± 1***	126
96	6	331 ± 9	87 ± 6	1.09 ± 0.34*	723 ± 186**	151 ± 12*	35 ± 3**	115
168	6	341 ± 8	100 ± 18	0.82 ± 0.20**	747 ± 181**	109 ± 4	21 ± 1	87
216 (9 days)	6	336 ± 14	106 ± 7	1.31 ± 0.33**	998 ± 185**	131 ± 15	30 ± 3*	101
264 (11 days)	6	348 ± 4	123 ± 14	1.34 ± 0.32**	1086 ± 200***	124 ± 7	30 ± 1**	94
336 (14 days)	6	323 ± 8	115 ± 10	0.96 ± 0.30*	865 ± 242*	111 ± 5	31 ± 3*	80
504 (21 days)	6	343 ± 9	99 ± 9	0.66 ± 0.17**	642 ± 169**	102 ± 4	23 ± 2	79
672 (28 days)	6	358 ± 11	124 ± 6	0.52 ± 0.20	555 ± 198*	94 ± 9	22 ± 2	72
840 (35 days)	6	361 ± 17	120 ± 13	0.48 ± 0.08**	589 ± 118**	82 ± 7*	18 ± 1*	64
1008 (42 days)	6	396 ± 6	122 ± 14	0.24 ± 0.09	213 ± 84	111 ± 3	26 ± 1*	85
1176 (49 days)	6	363 ± 12	120 ± 10	0.14 ± 0.11	113 ± 36	109 ± 5	25 ± 1	84

Values are mean ± s.e.mean. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$, values are significantly different from those of untreated control group.

control values for the remainder of the experiment, with an exception at 336 h (14 days) after injection when there was a marked fall in uterine water content.

Effect on tamoxifen on oestrogen receptor levels (Figure 1)

Twenty four hours after tamoxifen treatment cytosolic oestrogen receptor levels had decreased markedly compared with control values, remaining depressed until 504 h (21 days) after injection when the levels began to increase toward control values.

The concentration of nuclear receptors reached a maximum at 27 h, with two further minor peaks occurring at 96 h and 336 h (14 days) after injection ($P < 0.05$ compared with preceding value). By 504 h (21 days) nuclear receptor levels had declined to a plateau, which was still above control values and this was maintained until the cessation of receptor measurements at 1176 h (49 days).

Discussion

The increases in uterine weight in response to a single dose of tamoxifen were not so sustained as those seen with uterine blood flow. A notable feature of the uterine weight changes was a fall in both wet and dry weight to below pretreatment levels at 840 h (35 days), possibly indicating a loss of uterine ground substance. All of the parameters showed two major peaks (in the case of uterine wet weight the second peak was less prominent), which is consistent with the results of Fromson *et al.* (1973) who found that tamoxifen absorption led to 2 serum maxima.

The results of this study show that tamoxifen caused a maximal depletion of cytosolic receptor 24 h after injection (when according to Bowman *et al.* (1982), serum concentrations of tamoxifen and monohydroxytamoxifen are at their highest). In general, changes in nuclear receptor were inversely related to the cytosol receptor changes. Bowman *et al.* (1982) found a similar receptor distribution, although this group used a slightly lower dose of tamoxifen (receptor dynamics are dose-related, Agarwal *et al.*, 1982) and administered it intraperitoneally. The previous study was completed 16 days after tamoxifen dosing (receptor levels having returned to control values at this time). Kirchhoff *et al.* (1983) also observed a prolonged depletion of cytosolic receptor concentrations after application of the tamoxifen metabolite monohydroxytamoxifen (the latter has a higher affinity for the oestrogen receptor than tamoxifen (Lieberman *et al.*, 1983; Katzenellenbogen *et al.*, 1983)).

Between 24 h and 96 h after tamoxifen injection, cytosolic receptor levels did increase (but were still only approximately 36% of the pretreatment value), indicating that some cytosolic replenishment was occurring. After this time receptor levels remained approximately constant, while the number of nuclear receptors remained above control. This may be indicative of an 'equilibrum' situation whereby continuing cytosolic receptor translocation maintains an elevated nuclear receptor population.

Although tamoxifen has a long biological half-life (Fromson *et al.* (1973) found it to be 10 days after a single 1.8 mg kg^{-1} i.p. dose), it seems unlikely that the serum concentration of drug or active metabolite can totally account for the extended duration of action observed in this study. The so-called anti-oestrogen binding site (Sutherland *et al.*, 1980) may be of relevance in this context, for if tamoxifen or its metabolites were bound at these sites in the uterus, the resultant effect may be a concentrated 'pool' of anti-oestrogen in the uterus even though serum levels had become negligible. In terms of the relationship between uterine blood flow and nuclear receptor stimulation by tamoxifen, there does appear to be an initial synchrony in that receptor concentration is maximal 27 h after tamoxifen injection and blood flow is also increasing to reach its first peak 30 h after injection. However, after this blood flow again begins to increase whilst the nuclear receptor concentration declines. Possibly the later stages of the hyperaemic response are as a result of new blood vessel growth.

The changes in wet weight seen in this study closely correspond to the results of Koseki *et al.* (1977), although their study ceased 100 h after tamoxifen administration. Uterine wet weight was at a maximum 21 h after nuclear receptor levels peaked, receptor levels then declined rapidly whilst wet weight decreased more slowly. Some workers (Castracane & Jordan, 1973; Koseki *et al.*, 1977) have suggested that it is the length of time of nuclear occupancy which is critical when considering the relationship between a uterotrophic response and receptor distribution, in which case water imbibition may well be an oestrogen receptor-mediated response. It this is so, perhaps the lag between receptor stimulation and uterotrophic response indicates that the receptor interaction constitutes only the first step of the response, which leads to a cascade of events terminating in, for example, histamine release. (Although work in this laboratory (Marshall & Senior, 1986) indicated that, in fact, tamoxifen is much less dependent on histamine as a mediator than oestradiol). Alternatively, if the wet weight response is not the eventuality of the classical oestrogen receptor interaction, it would seem that tamoxifen (or its metabolites) is capable of stimulating this other mechanism. A non-genomic pathway has been proposed by Tchernitchin (1979, 1983) involving eosinophil migration and the triphenylethylene anti-oestogen nafoxidine has been shown to induce eosinophil migration (Galand *et al.*, 1984).

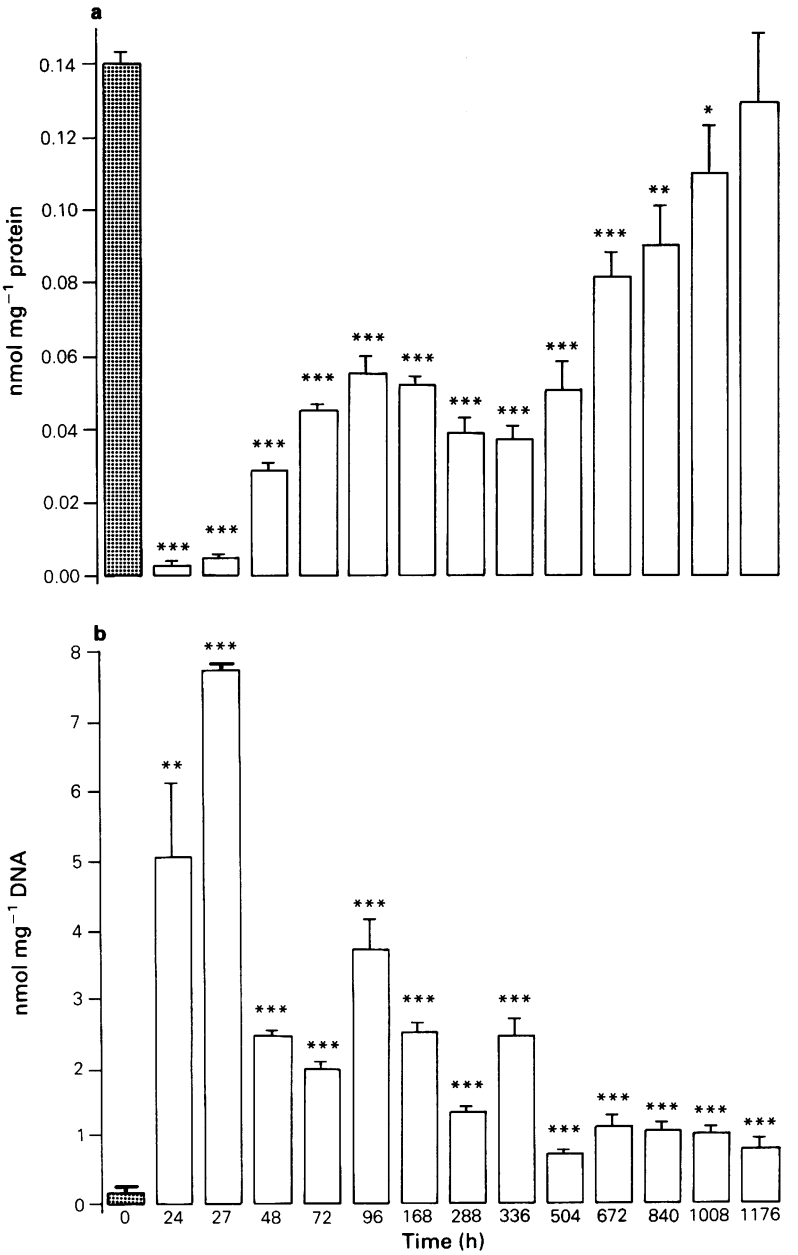

Figure 1 The effect of a single subcutaneous injection of tamoxifen (1 mg kg^{-1}) on cytosolic (a) and nuclear (b) oestrogen receptor distribution in mature ovariectomised rats. The results are shown at various times after injection. Each column represents mean of 3–6 determinations, with 6 uteri per determination; vertical lines show s.e.mean. Shaded columns represent control group and open columns treated groups. *$P < 0.05$, **$P < 0.005$, ***$P < 0.001$, values are significantly different from those of untreated control group.

Tamoxifen or its metabolites did effect increases in uterine dry weight which initially followed increases in nuclear receptor. However, some elevation in dry weight was maintained whilst the concentration of nuclear receptor was declining, although, Clark & Peck (1976) demonstrated that only a small proportion of receptor sites need to be retained within the nucleus to maintain uterine growth. The later increases in dry weight seen could also be attributable to stimulation of the so-called type II nuclear receptors (Clark & Markaverich, 1981; 1982) which are associated with true uterine growth. Lyttle *et al.* (1984) have postulated that eosinophils are the source of nuclear type II receptors. Changes in dry weight could also reflect increases in other proteins such as the progesterone receptor. Tamoxifen has been shown to be capable of stimulating progesterone receptor synthesis (Jordan & Gosden, 1983). Bulger & Kupfer (1976) demonstrated that tamoxifen could induce ornithine decarboxylase activity which in turn could stimulate further protein synthesis.

In conclusion, it would seem that the classical genomic oestrogen receptor interaction may not fully account for the uterotrophic events seen after tamoxifen administration. (Although, it should be noted that increases in receptor concentration may not necessarily reflect the quality of the receptor binding – it is possible that the tamoxifen-receptor conformation is aberrant in some way). Alternatively, tamoxifen or one of its metabolites (studies with antioestrogens *in vivo* have revealed that their activity depends upon the composite activity of the various forms of the drug (Katzenellenbogen *et al.*, 1983)) may be mimicking the action of oestradiol by, for example, releasing mediators.

This work was supported by a grant to K.M. from the Pharmaceutical Society of Great Britain.

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(Received March 31, 1987.
Revised June 4, 1987.
Accepted June 16, 1987.)