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Modification of the rat uterine response to oestrogen and tamoxifen by thromboxane antagonists

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1 The thromboxane receptor antagonists EP092, AH23848 and BM 13.505 were used to investigate the role of thromboxane in the uterotrophic response to oestradiol and tamoxifen.

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 Only EP092 potentiated the hyperaemic response to oestrogen but all three thromboxane antagonists inhibited oestradiol-stimulated uterine growth. This inhibition was accompanied by a decrease in nuclear oestrogen receptor concentration.

4 The uterotrophic response to tamoxifen was unaffected by the thromboxane antagonists.

5 The mechanism by which the thromboxane antagonists may be exerting their growth inhibitory effect is discussed, although, whether this effect can be attributed to blockade of thromboxane receptors or to some other mechanism is not clear from this study.

Introduction

As a time delay is apparent between the administration of oestradiol and the onset of the early uterotrophic response, a number of mediators have been implicated in the induction of the response to the steroid. Much experimental evidence points to a prostaglandin involvement in oestrogen-induced uterine events (Ryan *et al.*, 1974; Phaily & Senior, 1978). Various groups have reported increases in uterine prostaglandin synthesis after oestrogen exposure (Castracane & Jordan, 1975; Ham *et al.*, 1975; Poyser & Scott, 1980; Poyser, 1983); indeed uterine prostaglandin synthetase activity correlates with ovarian oestradiol output (Poyser & Scott, 1980). Since rat uterine homogenates are capable of generating both thromboxane A_2 and prostacyclin (Brown & Poyser, 1985), it is likely that the balance between the two is crucial to many reproductive processes.

In this study the contribution of thromboxane to the uterotrophic response induced by oestrogen and the non-steroidal anti-oestrogen, tamoxifen (which in the rat behaves as a partial agonist), was investigated by use of thromboxane receptor blocking drugs, namely EP092, AH23848 and the non-prostanoid antagonist BM 13.505. These compounds have proven receptor blocking activity (Armstrong *et al.*, 1984; 1985; Brittain *et al.*, 1985; Stegmeier *et al.*, 1986).

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 in this study were bilaterally ovariectomized at least 21 days before further experimentation and were randomly assigned to groups. All the animals used were of similar body weight (around 330 g); there was no significant difference in weights within groups or between 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 Sc^{46} and suspended in 10% (w/v) dextran containing 0.1% (w/v) Tween 80. Approximately 100,000 microspheres were injected into the left ventricle at a steady rate for 10 s. Uterine blood flows were calculated from wet weight of tissue. In all cases blood flow between the adrenals and kidneys was balanced, indicating a uniform distribution of microspheres. In the control group cardiac output was 113 ± 13 ml min^{-1} and was unaffected by any of the treatments as was heart rate (375 ± 15 beats min^{-1}). When dry weights are quoted the tissue was dried until the weight remained constant.

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

Homogenization, cytosol and nuclear pellet preparation and 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. The exchange conditions employed were found to be optimal with respect to maximal exchange with minimal loss of binding sites. 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 [^{3}H]-oestradiol ([2,4,6,7-^{3}H]-oestradiol, 85–110 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 oestogen 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 (45% efficiency).

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* (King & Greene, 1984). The nuclear or activated form of the receptor remains in the nuclear compartment after fractionization. Although receptor localization is important, in terms of this study, 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 compartmentalize the oestrogen receptor.

Drug administration

Oestradiol (oestra-1,3,5-(10)-triene-3,17β-diol; BDH, Poole) was dissolved in a 10% (v/v) solution of propylene glycol and was administered intravenously (i.v.) at a dose level of 0.5 μg 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, 24 h before measurements were made, at a dose level of 1 mg kg^{-1} as previously described (Majid & Senior, 1982). Serum levels of tamoxifen have also been shown to peak at this time (Bowman *et al.*, 1982). The solvents used did not have any effect on the parameters measured and these results have been grouped as controls. EP092 ((±)-5-endo(6'-carboxyhex-2'Z-enyl)-6-exo-(1''-[N-(phenylthiocarbamoyl)-hydrazono]-ethyl-bicyclo[2,2,1] heptane) (Pharmacology Dept., University of Edinburgh) was dissolved in absolute alcohol (5 mg ml^{-1}) plus 2.5 μl M sodium hydroxide per mg EP092; the ethanol was then evaporated under nitrogen and the resultant sodium salt was dissolved in aqueous sodium hydroxide (M/400)/sodium chloride (0.9% w/v). EP092 was given 1 h 30 min and 3 h 10 min before measurements were made. AH23848 ([1α(Z),2β, 5α]-(±)-7-[5-[[(1,1'-biphenyl)-4-yl]methoxy]-2-(4-morpholinyl)-3-oxo-cyclopentyl]-4-heptenoic acid) (Glaxo Group Research) was dissolved in 1% w/v sodium bicarbonate in sodium chloride (0.9% w/v). BM 13.505 (4-(2-(4-chlorobenzenesulphonamide)-ethyl)-benzene acetic acid) (Boehringer Mannheim, GmbH) was obtained in a stock solution of BM 13.505 100 mg, meglumin 65 mg; mannitol 164 mg; Tritriplex III 1 mg in water for injection to 5 ml, all subsequent dilutions were made in water for injection. Both AH23848 (10 mg kg^{-1}) and BM 13.505 (20 mg kg^{-1}) were administered intravenously 3 h 10 min before measurements were made. The doses of the thromboxane antagonists were chosen after preliminary studies which indicated that at these dosage levels the uterotrophic response was significantly altered.

Statistical analysis

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

Results

In the absence of any exogenous oestradiol the thromboxane antagonists had no significant effect on the parameters studied.

Effect of EP092, AH23848 and BM 13.505 on oestradiol stimulated blood flow (Table 1)

The vehicles used for the thromboxane antagonists had no effect upon the oestradiol response. The addition of EP092 to the oestradiol regimen significantly enhanced the blood flow response. AH23848 and BM 13.505 had no effect on the oestradiol-induced hyperaemia. At these doses the antagonists had no effect on cardiovascular function or blood flow to other organs.

Effect of EP092, AH23848 and BM 13.505 on oestradiol-stimulated uterine weight (Table 1)

BM 13.505 reduced the uterine water imbibition response to oestradiol. All three antagonists effected a significant decrease in oestradiol-induced uterine dry weight.

Effect of EP092, AH23848 and BM 13.505 on tamoxifen-stimulated blood flow (Table 2)

No effect upon tamoxifen-stimulated uterine blood flow was observed in the presence of the thromboxane antagonists or their vehicles, nor did the antagonists affect the cardiovascular and regional organ blood flow in response to tamoxifen.

Effect of EP092, AH23848 and BM 13.505 on tamoxifen-stimulated uterine weight (Table 2)

The increases in tamoxifen-stimulated wet and dry uterine weight were unaffected by the presence of the thromboxane antagonists.

Table 1 The effect of EP092 (10 mg kg^{-1} i.v., 2 doses) given 10 min before and 1 h 30 min after oestradiol, AH23848 (2.5 mg kg^{-1} i.v., single dose) and BM 13.505 (20 mg kg^{-1} i.v., single dose) given 10 min before oestradiol on uterine blood flow and weight

Pretreatment	*No. of rats*	*Uterine blood flow* (ml min^{-1} 100 g^{-1})	*Uterine weight* (mg) *Wet*	*Dry*
None (control)	7	37 ± 10	75 ± 8	17 ± 1
Oestradiol alone	8	536 ± 102†††	99 ± 5†††	27 ± 1†††
Oestradiol + EP092 vehicle	7	540 ± 96	100 ± 5	26 ± 1
Oestradiol + EP092	12	889 ± 119*	92 ± 12	22 ± 1*
Oestradiol + AH23848 vehicle	8	542 ± 90	97 ± 6	27 ± 1
Oestradiol + AH23848	7	624 ± 67	91 ± 5	20 ± 1***
Oestradiol + BM 13.505 vehicle	6	560 ± 13	99 ± 4	24 ± 1
Oestradiol + BM 13.505	4	562 ± 85	81 ± 5*	16 ± 1.5**

Measurements were made 3 h after oestradiol (0.5 μg kg^{-1} i.v., single dose) administration.
Values are mean ± s.e.mean; ††† $P < 0.001$ values are significantly different from those of control group; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$ values are significantly different from those of oestradiol + vehicle group.

Table 2 The effect of EP092 (10 mg kg^{-1} i.v., 2 doses) given 1 h 30 min and 3 h 10 min before tamoxifen, and AH23848 (2.5 mg kg^{-1} i.v., single dose) and BM 13.505 (20 mg kg^{-1} i.v., single dose) given 3 h 10 min before tamoxifen on uterine blood flow and weight

Pretreatment	No. of rats	Uterine blood flow (ml min^{-1} 100 g^{-1})	Uterine weight (mg) Wet	Dry
None (control)	7	37 ± 10	75 ± 8	17 ± 1
Tamoxifen alone	7	123 ± 26†	136 ± 7††	24 ± 1†
Tamoxifen + EP092 vehicle	7	105 ± 16	129 ± 8	23 ± 2
Tamoxifen + EP092	9	138 ± 14	138 ± 12	25 ± 2
Tamoxifen + AH23848 vehicle	7	120 ± 13	140 ± 6	24 ± 1
Tamoxifen + AH23848	6	112 ± 17	143 ± 11	25 ± 2
Tamoxifen + BM 13.505 vehicle	6	107 ± 11	131 ± 7	23 ± 1
Tamoxifen + BM 13.505	6	122 ± 15	133 ± 6	24 ± 1

Measurements were made 24 h after tamoxifen (1 mg kg^{-1} s.c., single dose) administration.
Values are mean ± s.e.mean; † $P < 0.05$; †† $P < 0.005$ values are significantly different from those of control group.

Effect of EP092, AH23848 and BM 13.505 on oestrogen receptor levels (Figure 1)

The vehicles used had no effect upon oestrogen receptor levels. Three hours after administration of oestradiol alone there was a decrease in cytosolic receptor concentration which was accompanied by an increase in nuclear receptor concentration compared to control. EP092 pretreatment induced a slight increase in cytosolic oestrogen receptor whilst causing a reduction in the number of oestrogen receptors in the nuclear compartment on comparison with oestradiol alone. Pretreatment with AH23848 had a similar effect but the increase in cytosolic receptor concentration was significant when compared with the oestradiol alone response. BM 13.505 had little effect on the oestradiol response with respect to cytosolic receptor, but this compound caused a marked reduction in nuclear receptor content. As the thromboxane antagonists had no apparent effect on the uterotrophic response to tamoxifen, equivalent receptor measurements were not made.

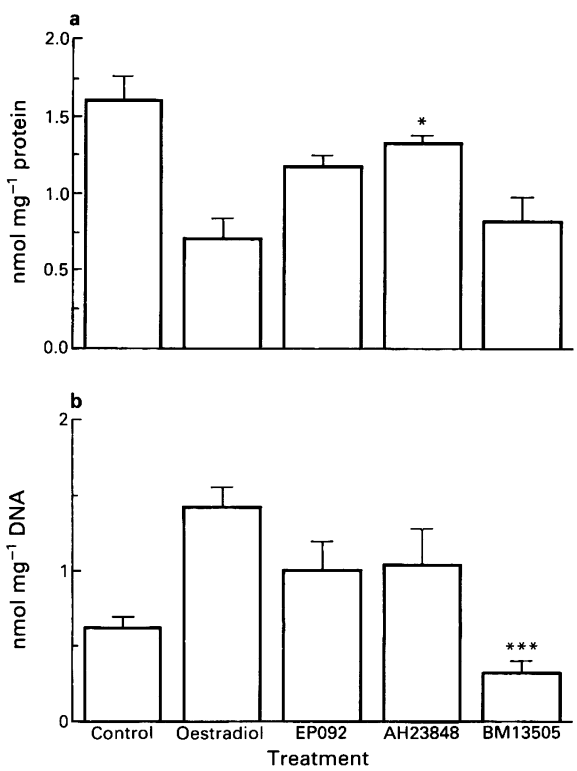

Figure 1 The effect of EP092 (10 mg kg^{-1}, i.v., 2 doses) given 10 min before and 1 h 30 min after oestradiol, AH23848 (2.5 mg kg^{-1}, i.v., single dose) and BM 13.505 (20 mg kg^{-1}, i.v., single dose) given 10 min before oestradiol on cytosolic (a) and nuclear (b) oestrogen receptor distribution in mature ovarectomized rats. Measurements were made 3 h after oestradiol (0.5 μg kg^{-1}, i.v., single dose) administration. Each column represents mean of 3–6 determinations, with 6 uteri per determination; vertical bars show s.e.mean. * $P < 0.05$, *** $P < 0.001$ values are significantly different from those of oestradiol alone group.

Discussion

Stimulation of prostaglandin synthesis by oestradiol has been widely accepted (Ramwell *et al.*, 1977). An oestradiol-induced rise in the thromboxane content of the ovariectomized rat uterus has also been reported (Jouanen *et al.*, 1985). Ramwell *et al.* (1977) suggested that much of the apparent stimulation of thromboxane synthesis by oestradiol may occur indirectly as a result of hyperaemia. Hyperaemia increases the blood volume in uterine tissues which consequently elevates the uterine platelet population. A numerical rise in platelet content alone may be sufficient to explain the enhanced thromboxane synthesis. However, direct oestrogen-stimulated thromboxane production by uterine myometrial and, to a greater extent, uterine endometrial tissue of ovariectomized rats has been reported (Brown & Poyser, 1985).

The cited evidence which suggests that oestradiol increases uterine thromboxane content, whether by *de novo* synthesis, increased uptake from other sources, enhanced release or diminished metabolic degradation, suggests that the thromboxane antagonists would enhance the oestradiol-induced hyperaemia through an ability to antagonize the powerful vasoconstrictor activity of thromboxane. The doses of the antagonists used in this work were selected as it has been demonstrated that they lie within the range of thromboxane antagonist activity (Armstrong *et al.*, 1984; 1985; Brittain *et al.*, 1985; Stegmeier *et al.*, 1986). However, in this study only EP092 was observed to elicit what would be expected as the classical effect of a thromboxane antagonist. Neither AH23848 nor BM 13.505 had any impact on the oestrogen-induced hyperaemia; this may be attributable to the fact that both these compounds display partial agonist activity (Brittain *et al.*, 1985; Patscheke *et al.*, 1987).

Only BM 13.505 caused a significant reduction in oestradiol-stimulated uterine wet weight although a similar trend was apparent with EP092 and AH23848. It has been suggested (Tchernitchin, 1979; 1983) that water imbibition is the result of eosinophilia. Therefore, it may be possible that thromboxane plays an effector role in the oestrogen induced cascade of events initiated by eosinophils; alternatively it may exert an anti-chemotactic influence on the eosinophils so as to prevent the initial migration to the uterus.

All the thromboxane antagonists caused a reduction in oestradiol-induced uterine growth. There is much evidence to substantiate a role for thromboxane in cell proliferation. Thromboxane A_2 is thought to be involved in initiating liver regeneration after partial hepatectomy in the rat (Kanzaki *et al.*, 1979), a tissue in which oestrogen receptors have been detected (Eisenfeld & Aten, 1979). Thromboxane A_2 has also been reported to enhance proliferation of B_{16} melanoma cells (Mahmud *et al.*, 1984); Honn *et al.* (1982) suggested a bidirectional control of tumour cell proliferation by the endogenous or exogenous thromboxane/prostacyclin balance; they demonstrated that thromboxane A_2 and its metabolites and analogues, stimulate tumour growth. It is possible that the stimulatory effect of thromboxane A_2 on cell growth may be attributed to an inhibitory action on adenylate cyclase. (Thromboxane B_2 causes a reduction in the basal level of intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) in B16a tumour cells: Honn *et al.*, 1982). However, cyclic AMP-independent inhibition of DNA synthesis has been observed by Wiley *et al.* (1983).

In the present study it could be presumed that the inhibitory effect of all three compounds on oestradiol-stimulated uterine dry weight was by virtue of thromboxane antagonism. However, no conclusive evidence can be presented to substantiate this, except that all three compounds have been shown to exhibit thromboxane receptor blocking activity on other smooth muscle preparations (vascular and non-vascular) and on platelets. In this instance, doubt is cast as to the exact nature of the dry weight-inhibitory effect on uterus since the classical vascular response to thromboxane antagonism, namely vasodilatation, was not uniformly observed with all three compounds.

Assuming that the mitogenic effects of oestrogen are at least partly dependent on the classical genomic response, the decreases in uterine dry weight seen in the presence of the thromboxane antagonists should be reflected by decreased nuclear receptor binding. This was in fact seen. This may be due to a direct interaction with the oestrogen receptor; alternatively, it could be an indirect effect, i.e. oestradiol was prevented from binding to its receptor in the presence of the thromboxane antagonists. Unfortunately, the quality of binding taking place cannot be assessed by the methodology employed in this work. The apparent loss of receptor observed as a result of pretreatment with BM 13.505 is suggestive of receptor processing which is associated with irreversible degradation of the activated receptor resulting in a diminution of functional receptor number (Horwitz *et al.*, 1978).

It is well-established that tamoxifen exhibits partial agonist properties in the rat uterus (Harper & Walpole, 1967). The agonist effect of tamoxifen on rat uterus has been demonstrated in this laboratory (Marshall & Senior, 1987), as have differences between the oestradiol- and tamoxifen-evoked responses (Marshall & Senior, 1986; 1989). In these experiments the thromboxane antagonists did not significantly alter the tamoxifen response. Tamoxifen is able to bind to the oestrogen receptor, although the resulting complex is thought to be in some way aberrant, which affects the interaction with the genome. It is possible that the conformational differences between the oestrogen/oestrogen receptor complex and the tamoxifen/oestrogen receptor complex may make the latter less susceptible to the effects of thromboxane receptor antagonists, hence the lack of modification of the uterine response. Alternatively, this discordance may be due to non-genomic mechanisms, as cell proliferation is also regulated by various growth factors such as platelet derived growth factor. Oestrogen and antioestrogen have opposing effects on peptide secretion (Dickson & Lippman, 1987). Therefore, it is possible that EP092, AH23848 and BM 13.505 are able to influence the factors produced as a result of oestrogen stimulation (hence the modification of the uterine weight response) but the antioestrogen evoked factors are not susceptible to their action.

In conclusion, the results of this study again point to diverging mechanisms of action between oestrogen and tamoxifen. From the results obtained in the oestradiol-stimulated uterus, it would seem that thromboxane may be a mediator of the early uterotrophic response. The susceptibility of the oestrogen weight response to inhibition by thromboxane antagonists is interesting as is the mechanism by which they exert this effect. Could the results of this work be extrapolated to other tissues where growth is hormone-dependent? We are accumulating evidence which indicates that the thromboxane antagonists inhibit growth of human derived hormone-dependent cell lines *in vitro*.

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