Unsaturated fatty acids as endogenous inhibitors of tamoxifen binding to anti-oestrogen-binding sites

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It is known that triphenylethylene anti-oestrogens such as tamoxifen bind to specific high-affinity anti-oestrogen-binding sites, which are distinct from oestrogen receptors. These binding sites are widely distributed in human and animal tissues, but their function and endogenous ligands are unknown. By using [^3H]tamoxifen and a rat liver microsomal fraction, a radio-ligand-binding assay was developed in an attempt to identify endogenous ligands for the anti-oestrogen-binding sites in the rat. An ether extract of rat serum inhibited [^3H]tamoxifen binding to rat liver binding sites in a dose-dependent manner. Identification of the active serum constituents that inhibited [^3H]tamoxifen binding was achieved by g.l.c.–mass spectrometry after preliminary purification of a rat serum extract by silica-gel t.l.c. Three unsaturated fatty acids (oleic, linoleic and arachidonic) accounted for about 50% of the total inhibiting activity of the serum extract. The concentrations of these fatty acids required to inhibit [^3H]tamoxifen binding were in the range of 10–100 μM , comparable with those found in the rat circulation under physiological conditions. Saturated fatty acids present in rat serum (palmitic and stearic) did not inhibit [^3H]tamoxifen binding. A survey of other fatty acids revealed that, in general, unsaturated fatty acids were far more potent than saturated fatty acids in inhibiting [^3H]tamoxifen binding. These studies demonstrate that unsaturated fatty acids are quantitatively the most important circulating inhibitors of [^3H]tamoxifen binding to the anti-oestrogen-binding sites. The biological significance of their interaction with these sites, however, remains to be clarified.

INTRODUCTION

It is generally accepted that the triphenylethylene anti-oestrogens such as tamoxifen and clomiphene exert their anti-oestrogenic effects by binding to oestrogen receptors (Katzenellenbogen *et al.*, 1979; Jordan, 1985). Specific high-affinity binding sites for the triphenylethylene anti-oestrogens, distinct from oestrogen receptors, have also been identified in many normal tissues in both animals (Sutherland *et al.*, 1980; Gulino & Pasqualini, 1982; Sudo *et al.*, 1983; Faye *et al.*, 1983) and man (Kon, 1983), as well as in human cancer cells (Miller & Katzenellenbogen, 1983; Watts *et al.*, 1984; Kon, 1985). The function of these binding sites is currently unknown, and their possible role in mediating the biological effects of non-steroidal anti-oestrogens remains controversial (Miller & Katzenellenbogen, 1983; Murphy & Sutherland, 1983; Sheen *et al.*, 1985). The wide distribution of these high-affinity binding sites suggests that substances structurally similar to anti-oestrogens may be produced endogenously. Identification of these endogenous ligands may provide information on the physiological role of the anti-oestrogen-binding sites. Several studies indicate that extracts of rat liver (Clark *et al.*, 1983), chicken liver and serum (Murphy *et al.*, 1984) and human serum (Murphy *et al.*, 1985) contain substances that inhibit binding of [^3H]tamoxifen to the anti-oestrogen-binding sites. Evidence has also been presented to suggest that oxygenated metabolites of cholesterol (Murphy *et al.*, 1985) and histamine-like substances (Brandes *et al.*, 1985) might represent

endogenous ligands. The studies reported here were carried out to identify possible endogenous ligands in rat serum. They demonstrate that unsaturated fatty acids present in rat serum inhibit [^3H]tamoxifen binding at physiological concentrations and account for a major proportion of the total inhibitory activity detectable in rat serum extracts.

MATERIALS AND METHODS

Chemicals

[*N*-methyl- ^3H]Tamoxifen (87 Ci/mmol) was obtained from Amersham International and stored in ethanol at -20°C protected from light. Nafoxidine hydrochloride and CI 628 were graciously provided by Upjohn Co. Tamoxifen citrate, clomiphene citrate, diethylstilboestrol, fatty acids, fatty acid methyl esters and steroids were obtained from Sigma Chemical Co. 7-Oxocholesterol was obtained from Steraloids. All other chemicals were of analytical grade and were obtained from conventional sources.

Tissue preparation

Livers were removed from 250–300 g female Sprague-Dawley rats under diethyl ether anaesthesia and homogenized in 12 vol. of ice-cold buffer [10 mM-Tris/HCl, 1.5 mM-EDTA, 20 mM-sodium molybdate, 10% (v/v) glycerol, pH 7.5 at 4°C] with an Ultra-Turrax homogenizer (full speed for 30s). The homogenate was centrifuged at 20000 *g* for 30 min and the supernatant was stored at -70°C .

The trivial names used are: tamoxifen, 1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenylbut-1(*Z*)-ene; clomiphene, 1-[4-(2-diethylaminoethoxy)phenyl]-1,2-diphenyl-2-chloroethylene; nafoxidine, 1-[2-[4-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl]pyrrolidine hydrochloride; CI 628, α -(4-pyrrolidinoethoxy)phenyl-4-methoxy- α' -nitrostilbene.

Ligand-binding assay

The 20000 *g* supernatant prepared from rat liver as described above was diluted 10-fold with buffer (final protein concn. 0.5–1.0 mg/ml) and incubated with approx. 50000 c.p.m. (1 nM) of [³H]tamoxifen (dissolved in 25 μ l of ethanol) in the absence or presence of various competing ligands (dissolved in 25 μ l of ethanol) in a final volume of 550 μ l. All assay tubes contained 1 μ M-diethylstilboestrol to eliminate binding to oestrogen receptors. After incubation for 16 h at 4 °C, 0.25 ml of dextran-coated charcoal (10 mM-Tris/HCl, 10% glycerol, 0.25% Norit A charcoal, 0.025% dextran T70, pH 7.5 at 4 °C) was added. The charcoal was pelleted by centrifugation at 1500 *g* for 15 min. Supernatants were counted for radioactivity in 7 ml of scintillant [containing 5 g of 2,5-diphenyloxazole, 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 300 ml of Triton X-100 and 700 ml of toluene (per litre)] in a liquid-scintillation spectrometer with a counting efficiency of approx. 50%.

T.l.c. of rat serum extract

Serum obtained from female Sprague–Dawley rats weighing 250–300 g was extracted with 4 vol. of diethyl ether. The ether extract was dried under N₂, redissolved in a small volume of chloroform and applied to a 20 cm $\times$ 20 cm silica-gel plate (AL SIL G; Whatman). After chromatography with hexane/diethyl ether/acetic acid (70:30:1, by vol.), or hexane/diethyl ether/acetic acid (90:10:1, by vol.), or benzene/acetone (4:1, v/v), 1 cm segments were scraped off the plate and the scrapings were eluted with 2 ml of diethyl ether. The eluates were dried under N₂ and redissolved in ethanol for the ligand-binding assay. The positions of authentic lipid standards were identified either by spraying the chromatograms with 5% (v/v) H₂SO₄ and heating to 180 °C for 30 min or by exposure to iodine vapour.

G.l.c. and mass spectrometry

Eluates of segments from thin-layer chromatograms which inhibited [³H]tamoxifen binding were dried under N₂ and treated with 1 ml of 14% (v/v) BF₃ in methanol for 5 min at 100 °C (Morrison & Smith, 1964). After addition of 1 ml of water, the fatty acid methyl esters were extracted into 2 vol. of pentane, dried under N₂ and redissolved in chloroform or hexane for g.l.c. and mass-spectrometric analysis. G.l.c. was carried out on a Varian model 3400 apparatus with stainless-steel columns [outer diameter 3.2 mm ($\frac{1}{8}$ in), length 2 metres] containing 15% OV-275 on Chromosorb W (mesh 80–100). N₂ flow was 30 ml/min, and the column temperature was programmed to rise from 120 °C to 210 °C at a rate of 15 °C/min, followed by an isothermal programme at 210 °C. The methyl esters were identified by comparing their relative retention times with those of authentic compounds. Quantification was carried out with heptadecanoic acid as internal standard together with known amounts of authentic compounds. For mass spectrometry, a Hewlett–Packard gas chromatograph–mass spectrometer (model 5995) equipped for electron-impact ionization was used. Conditions were identical with those described above, except that helium was the carrier gas. The spectra of unknowns were compared with those of authentic compounds.

Other procedures

Procedures for saturation analysis (Scatchard, 1949) and gel filtration are detailed in the appropriate Figure legends. Protein was determined by the method of Lowry *et al.* (1951).

RESULTS

Fig. 1 shows the specificity of the assay system used to identify substances with the ability to inhibit binding of [³H]tamoxifen to the anti-oestrogen-binding site of rat liver. The triphenylethylene anti-oestrogens (tamoxifen, CI 628, nafoxidine and clomiphene) inhibited [³H]tamoxifen binding in a dose-dependent manner. Half-maximal inhibition was observed at concentrations between 2.3 nM (clomiphene) and 5.2 nM (tamoxifen). A variety of steroids, including corticosterone, cortisol, deoxycorticosterone, dehydroepiandrosterone, progesterone, testosterone, androstenedione, 17 β -oestradiol, oestrone, oestriol and cholesterol, did not inhibit binding at concentrations as high as 10 μ M. A diethyl ether extract of rat serum, on the other hand, gave a dose–response curve similar to that of the triphenylethylene derivatives, indicating that rat serum contains one or more ether-soluble substances that inhibit [³H]tamoxifen binding to the anti-oestrogen-binding site.

Fig. 2 shows the distribution of inhibiting activity of a diethyl ether extract of rat serum after fractionation by silica-gel t.l.c. with the solvent system hexane/diethyl ether/acetic acid (70:30:1, by vol.). One major peak of activity was observed at segments 11–13 (*R_F* 0.51–0.68), with a much smaller peak at segment 3 (*R_F* 0.0–0.06). The *R_F* value of the major peak was the same as that of marker non-esterified acids (stearic, oleic) but different from that of phosphatidylethanolamine, 7-oxocholesterol, cholesterol, linoleic acid methyl ester and cholesteryl oleate. The mobility of the inhibiting activity was also similar to that on non-esterified fatty acids in two other solvent systems, benzene/acetone (4:1, v/v) and hexane/diethyl ether/acetic acid (90:10:1, by vol.), in which the *R_F* values were 0.60–0.74 and 0.17–0.23 respectively. Furthermore, after gel filtration of rat serum on Sephadex G-100, inhibiting activity was found to be associated with albumin, a protein known to bind non-esterified fatty acid (result not shown).

The co-migration of inhibiting activity with fatty acids in several different chromatographic systems suggested that the inhibition of [³H]tamoxifen binding might be due to serum fatty acids. This was confirmed as follows.

First, derivatives of the active fraction recovered from silica-gel t.l.c. (segments 11–13, Fig. 2) were prepared with methanolic BF₃ and analysed by g.l.c. As shown in Fig. 3, seven peaks were observed. By comparing their relative retention times with those of authentic compounds, peaks 2, 3, 4, 5 and 6 were found to be palmitic acid (C_{16:0}), stearic acid (C_{18:0}), oleic acid (C_{18:1}), linoleic acid (C_{18:2}) and arachidonic acid (C_{20:4}) respectively. The identities of these peaks were further confirmed by mass spectrometry, by comparing their spectra with those of authentic compounds. Peaks 1 and 7 remain to be identified.

Secondly, the five major fatty acids identified in the active peak from t.l.c. were examined for their ability to inhibit [³H]tamoxifen binding. As shown in Fig. 4, oleic acid, linoleic acid and arachidonic acid inhibited

Fig. 1. Ligand specificity of anti-oestrogen-binding sites of rat liver

[³H]Tamoxifen was incubated with rat liver 20000 g supernatant in the absence and presence of various amounts of tamoxifen (○), CI 628 (□), nafoxidine (■), clomiphene (●), an ether extract of rat serum (▲), or the following steroids (△): corticosterone, cortisol, deoxycorticosterone, dehydroepiandrosterone, progesterone, testosterone, androstenedione, 17β-oestradiol, oestrone, oestriol and cholesterol. Details are as described in the text.

[³H]tamoxifen binding in a dose-dependent manner; these three fatty acids were approximately equipotent, achieving half-maximal inhibition at concentrations of 50–60 μM. On the other hand, palmitic acid and stearic acid did not inhibit [³H]tamoxifen binding at concentrations up to 125 μM. Higher concentrations of palmitic acid and stearic acid could not be tested because of insolubility.

From the above studies, it would appear that the ability of rat serum extracts to inhibit [³H]tamoxifen binding (Fig. 1) was due, at least in part, to fatty acids. To determine the quantitative contribution of fatty acids to this inhibitory activity, the amounts of oleic acid, linoleic acid and arachidonic acid present in rat serum extracts were measured by quantitative g.l.c. with heptadecanoic acid as internal standard in conjunction with known amounts of authentic compounds. In five separate experiments, it was observed that the oleic acid, linoleic acid and arachidonic acid contents of rat serum extracts accounted for 47, 48, 48, 49 and 53% of the total inhibitory activity detected. It is likely that other substances with [³H]tamoxifen-binding inhibitory activity exist. In view of the report that 7-oxocholesterol from human serum inhibited [³H]tamoxifen binding to chicken liver cytosol (Murphy *et al.*, 1985), rat serum extracts were analysed for the presence of this compound by g.l.c. However, no detectable amount of 7-oxocholesterol was found, and it would appear that, at least in rat serum, 7-oxocholesterol is not present in sufficient amounts to contribute significantly to the inhibitory activity observed.

The observation that, among the major circulating fatty acids, only those that are unsaturated (oleic, linoleic and arachidonic) inhibited [³H]tamoxifen binding suggested that unsaturation in some manner confers the

ability to inhibit [³H]tamoxifen binding. Accordingly a variety of other fatty acids were tested for their ability to inhibit [³H]tamoxifen binding. The results are shown in Table 1. Although several of the saturated fatty acids tested did inhibit [³H]tamoxifen binding, much higher concentrations of saturated fatty acids were required to achieve half-maximal inhibition of binding, compared with the unsaturated fatty acids. For the fatty acids tested, half-maximal inhibitory concentrations were in the range 45–95 μM for unsaturated fatty acids and 460–3200 μM for saturated fatty acids.

Several additional studies were carried out to characterize further the inhibitory effect of unsaturated fatty acids on [³H]tamoxifen binding. Fig. 5 shows the Scatchard analysis of [³H]tamoxifen binding to the anti-oestrogen-binding site of rat liver in the absence or presence of linoleic acid. The presence of linoleic acid did not significantly alter the horizontal intercept (number of binding sites), but clearly decreased the slope (binding affinity). The presence of 15 μM- or 30 μM-linoleic acid increased the apparent equilibrium dissociation constant (K_D) from 1.05 nM to 1.26 nM and 1.95 nM respectively.

Fig. 6 shows the distribution of radioactivity when the 20000 g rat liver supernatant was preincubated with [³H]tamoxifen and then fractionated on Sephadex G-25. Three major peaks of radioactivity were observed: one peak immediately after the void volume (V_0), a smaller peak just before the total bed volume (V_t) and a broad retarded peak emerging at about 3 bed volumes. When preincubation was carried out in the presence of a 1000-fold excess of non-radioactive tamoxifen (2.5 μM), the first peak was completely abolished, indicating that the void-volume peak contained the anti-oestrogen-binding sites. Linoleic acid, at a concentration of 250 μM, similarly selectively abolished this peak without signi-

Fig. 2. Distribution of $[^3\text{H}]$ tamoxifen-binding-inhibitory activity of rat serum extract after silica t.l.c.

The diethyl ether extract of rat serum was dried under N_2 , redissolved in a small volume of chloroform and applied to a silica-gel plate, and t.l.c. was carried out with the solvent system hexane/diethyl ether/acetic acid (70:30:1, by vol.), after which 1 cm segments were eluted with 2×2 ml of diethyl ether. The eluates were dried under N_2 and redissolved in ethanol for assay of inhibiting activity as described in the Materials and methods section. An extract of rat serum was used as an arbitrary standard to quantify the relative amounts of inhibiting activity found in individual segments of the chromatogram.

significantly affecting the other two peaks of radioactivity. Oleic acid had a similar effect (result not shown). The retarded peak of radioactivity (fractions 50–90) was completely adsorbable by activated charcoal and thus represented free $[^3\text{H}]$ tamoxifen. The nature of the second peak, which was not adsorbed by charcoal and not altered by the presence of non-radioactive tamoxifen or linoleic acid, was not clear.

DISCUSSION

The studies reported here attempted to identify, by means of an assay with specificity for triphenylethylene anti-oestrogens, endogenous ligands for anti-oestrogen-binding sites. It is clear that the binding of $[^3\text{H}]$ tamoxifen to rat liver anti-oestrogen-binding sites is inhibited by at least three unsaturated fatty acids (oleic, linoleic and arachidonic) present in rat serum. These three fatty acids together account for about 50% of the total inhibitory activity detected in rat serum extracts.

Several studies demonstrated that other endogenous

Fig. 3. G.l.c. of the active peak from t.l.c.

Segments 11–13 (Fig. 2) were eluted with diethyl ether. The eluate was dried under N_2 , a derivative was made by the BF_3 /methanol method (Morrison & Smith, 1964) and analysed on a Varian gas-liquid chromatograph as described in the Materials and methods section. For identification of the peaks observed, retention times relative to that of heptadecanoic acid (arrow) were compared with the relative retention times of authentic compounds. Quantification was carried out with heptadecanoic acid as internal standard in conjunction with known amounts of authentic compounds.

Fig. 4. Inhibition of $[^3\text{H}]$ tamoxifen binding by fatty acids

The assay for $[^3\text{H}]$ tamoxifen binding was carried out as described in the Materials and methods section in the absence or presence of various concentrations of stearic acid (○), palmitic acid (▼), oleic acid (▲), linoleic acid (■) and arachidonic acid (●).

Table 1. Concentrations of fatty acids required for half-maximal inhibition of [³H]tamoxifen binding to rat liver anti-oestrogen-binding sites

[³H]tamoxifen binding assays were set up as described in the Materials and methods section in the absence and presence of various concentrations of fatty acids. The concentrations of fatty acids giving half-maximal inhibition of specific binding were read from inhibition curves similar to those depicted in Fig. 4.

Fatty acid	Concn. giving half-maximal inhibition of binding (μM)
Unsaturated	
Docosa-4,7,10,13,16,19-hexaenoic	45
Oleic	50
Arachidonic	58
Linoleic	60
α -Linolenic	70
Docosa- <i>cis</i> -13-enoic	78
Palmitoleic	95
Saturated	
n-Dodecanoic	460
n-Decanoic	1700
n-Octanoic	2700
n-Hexanoic	3200
Palmitic	*
Stearic	*

*No inhibition of binding when tested to the limit of solubility (approx. 125 μM).

Fig. 5. Scatchard analysis of [³H]tamoxifen binding to anti-oestrogen-binding sites of rat liver

Rat liver 20000 *g* supernatant (0.5 ml; protein concn. 0.7 mg/ml) was incubated for 16 h at 4 °C with various concentrations of [³H]tamoxifen (0.25–5.0 nM) in the absence (▽) or presence (□) of 15 μM - or (△) 30 μM -linoleic acid. Separation of bound from free ligand was performed by using dextran-coated charcoal as described in the Materials and methods section. Specifically bound [³H]tamoxifen is the difference between binding obtained in the absence and presence of excess unlabelled tamoxifen (1.25 μM).

Fig. 6. Gel filtration of rat liver 20000 *g* supernatant prelabelled with [³H]tamoxifen

A 1 ml sample of rat liver 20000 *g* supernatant (protein concn. 0.7 mg/ml) was incubated at 4 °C for 16 h with approx. 250000 c.p.m. (5 nM) of [³H]tamoxifen in the absence (▲) or presence of 2.5 μM -tamoxifen (●) or 250 μM -linoleic acid (○). Chromatography was carried out on Sephadex G-25 column (1.6 cm × 30 cm) equilibrated with 10 mM-Tris/HCl (pH 7.5)/1.5 mM-EDTA/20 mM-sodium molybdate/10% (v/v) glycerol. Fractions (2.6 ml) were collected and counted for radioactivity.

substances such as 7-oxocholesterol (Murphy *et al.*, 1985) and histamine or histamine-like substances (Brandes *et al.*, 1985) also inhibited [3 H]tamoxifen binding to anti-oestrogen-binding sites. It is probably premature, however, to consider any of these substances or unsaturated fatty acids as endogenous ligands for these sites, because 7-oxocholesterol, histamine or fatty acids have not been shown to bind directly to the anti-oestrogen-binding site. Although the data presented in Figs. 5 and 6 may suggest that unsaturated fatty acids possibly compete with [3 H]tamoxifen for the anti-oestrogen-binding site, it had been difficult, in studies using [3 H]oleic acid (results not shown), to demonstrate direct competition between tamoxifen and oleic acid for these binding sites: a 1000-fold molar excess of non-radioactive tamoxifen failed to displace bound [3 H]oleic acid, as assessed by gel-filtration studies similar to those depicted in Fig. 6. In view of this observation, the inhibitory effect of unsaturated fatty acids on [3 H]tamoxifen binding to the anti-oestrogen-binding site may result from allosteric inhibition of [3 H]tamoxifen binding rather than from direct competition for the binding site. This possibility requires further exploration. Fatty acids have been reported to modulate other protein-ligand interactions as well, such as the binding of thyroxine to serum binding proteins (Chopra *et al.*, 1984) and the binding of oestrogens to rat α -foetoprotein (Vallette *et al.*, 1980) or to oestrogen receptors (Nunez *et al.*, 1983). In all these cases, the mechanism and significance of the fatty acid effect are unclear.

Although the inhibition of [3 H]tamoxifen binding by fatty acids is a phenomenon observed *in vitro*, several observations suggest that it may have biological significance. Firstly, unsaturated fatty acids are much more potent than saturated fatty acids in inhibiting [3 H]tamoxifen binding (Table 1), suggesting some degree of specificity. Secondly, unsaturated fatty acids appear to be quantitatively the most important inhibitors present in rat serum. Thirdly, the concentrations of unsaturated fatty acids required to inhibit binding (10–100 μ M) are in the physiological range; representative concentrations of serum fatty acids in the animals used in this study are as follows (mean $\pm$ S.D.): palmitic, 82 ± 30 μ M; stearic, 14 ± 2 μ M; oleic, 57 ± 17 μ M; linoleic, 84 ± 20 μ M; arachidonic, 27 ± 5 μ M.

The possible physiological significance of the fatty acid effect on the anti-oestrogen-binding site, however, is speculative at present. The anti-oestrogen-binding sites, whose physico-chemical characteristics (Faye *et al.*, 1983; Kon, 1983) and microsomal location (Sudo *et al.*, 1983) distinguish them from previously described fatty acid-binding proteins such as α -foetoprotein (Ruoslahti & Seppälä, 1979) and cytosolic fatty acid binding proteins (Glatz & Veerkamp, 1985), have been extensively studied, but their function remains obscure. The observation that fatty acids appear to interact with these sites raises the possibility that the anti-oestrogen-binding sites may mediate one or more of the diverse biological effects of unsaturated fatty acids, particularly those not known to be mediated via prostaglandins or other eicosanoids derived from unsaturated fatty acids. For example, unsaturated fatty acids have been found, on the basis of extensive epidemiological as well as experimental studies, to enhance the development and growth of a number of animal and human tumours (Committee on

Diet, Nutrition and Cancer, 1982; Byers & Graham, 1984), but the mechanism of this effect is entirely unknown. In particular, it is intriguing to note that, after the administration of the chemical carcinogen 7,12-dimethylbenzanthracene to rats, unsaturated fatty acids enhanced the development of mammary tumours (Hopkins *et al.*, 1981), whereas tamoxifen administration had the opposite effect (Jordan, 1985). Further examination of the potential role of the anti-oestrogen-binding site in the tumour-promoting and possibly other effects of unsaturated fatty acids seems worthwhile.

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