Microsomal epoxide hydrolase of rat liver is a subunit of the anti-oestrogen-binding site

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A tritiated photoaffinity labelling analogue of tamoxifen, [(2-azido-4-benzyl)-phenoxy]-*N*-ethylmorpholine (azido-MBPE), was used to identify the anti-oestrogen-binding site (AEBS) in rat liver tissue [Poirot, Chailleur, Fargin, Bayard and Faye (1990) *J. Biol. Chem.* **265**, 17039–17043]. UV irradiation of rat liver microsomal proteins incubated with tritiated azido-MBPE led to the characterization of two photolabelled proteins of molecular masses 40 and 50 kDa. The amino acid sequences of proteolytic products from the 50 kDa protein were identical with those from rat microsomal epoxide hydrolase (mEH). Treatment

of hepatocytes with anti-sense mRNA directed against mEH abolished AEBS in these cells. In addition we found that tamoxifen and *N*-morpholino-2-[4-(phenylmethyl)phenoxy]-ethanamine, a selective ligand of AEBS, were potent inhibitors of the catalytic hydration of styrene oxide by mEH. However, functional overexpression of the human mEH did not significantly modify the binding capacity of [³H]tamoxifen. Taken together, these results suggest that the 50 kDa protein, mEH, is necessary but not sufficient to reconstitute AEBS.

INTRODUCTION

The anti-oestrogen tamoxifen has been in use for more than 20 years to treat hormone-dependent breast cancers. It is now generally accepted that the pleiotropic activities exerted by tamoxifen cannot be explained solely by its binding to the oestrogen receptor (ER) [1] and support the existence of alternative ER-independent mechanisms of action [2–5]. Our laboratory previously characterized a high-affinity membranous binding site for triphenylethylenic anti-oestrogens, such as tamoxifen, that was unable to recognize oestradiol [6]. This site has been named AEBS, for anti-oestrogen-binding site.

Although AEBS was initially found in the cytosolic fraction of cells, Sutherland's group demonstrated that it was principally localized in the membranes of the endoplasmic reticulum [7,8]. Binding studies with tamoxifen showed that AEBS was detectable in neoplastic tissues as well as in normal tissues, and that it was present in nearly all tissues tested in mammals, although at different levels [9].

AEBS might be partly responsible for the antigrowth effects of tamoxifen. We first reported phenotypic evidence of this by isolating an AEBS-negative cell line Rtx-6 from MCF-7 cells (a human breast cancer cell line) that was resistant to tamoxifen. The Rtx-6 cells were similar to MCF-7 cells in their ER content and functionality, suggesting a correlative link between the lack of AEBS and the insensitivity of these cells to tamoxifen [10] and to a specific AEBS ligand [11,12]. Other studies have reported dose-dependent cytostatic and cytotoxic effects of anti-oestrogens on ER-negative lymphoid cells that contain a high level of microsomal AEBS, thus underlining the importance of AEBS in the mediation of growth control [12–14]. A diphenylmethane or

benzhydryl derivative of tamoxifen, *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine.HCl (DPPE), exhibited an affinity of 50 nM for AEBS but no affinity for ER [15,16]; it was antiproliferative on MCF-7 cells [11,17–20] but had no effect on the proliferation of Rtx-6. We have established a positive correlation between the affinity of DPPE-based derivatives for AEBS and their antiproliferative properties on MCF-7 cells. None of the AEBS ligands was able to affect AEBS-negative Rtx-6 cells [11]. A similar correlation has been reported by other groups on ER-negative cell lines for AEBS ligands of the triphenylethylenic series [13] or oxysterol series [21].

The first reports relating to the characterization of AEBS showed that it is a membranous protein of molecular mass 100–480 kDa [6,8]. We have previously shown by photoaffinity labelling with [(2-azido-4-benzyl)-phenoxy]-*N*-ethylmorpholine (azido-MBPE) that the rat liver microsomal binding site has a molecular mass of between 40 and 50 kDa [22]. Whether these two labelled proteins are part of the same complex or include other factors is currently unknown. We chose rat liver microsomes as the biological material for the purification because they contain 30–40 pmol of AEBS/mg of protein. Here we describe the identification of one subunit of AEBS.

MATERIALS AND METHODS

Materials

[³H]Tamoxifen (specific radioactivity 86 Ci/mmol) was from New England Nuclear, and unlabelled steroids and tamoxifen from Sigma. Tris, KCl, Na₂S₂O₃, glycerol, EDTA, propanediol and *N,N*'-dimethylformamide were from Merck. Monothioglycerol, 2-mercaptoethanol, taurine, Tween 20, SDS, styrene oxide and

Abbreviations used: azido-MBPE, [(2-azido-4-benzyl)-phenoxy]-*N*-ethylmorpholine; AEBS, anti-oestrogen-binding site; DPPE, *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine; ER, oestrogen receptor; MBPE, *N*-morpholino-2-[4-(phenylmethyl)phenoxy]ethanamine; mEH, microsomal epoxide hydrolase; RT-PCR, reverse transcriptase-mediated PCR.

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styrene diol were from Sigma; Bistris was from Serva. Azido-MBPE, [^3H]azido-MBPE (2.2 Ci/mmol) and *N*-morpholino-2-[4-(phenylmethyl)phenoxy]ethanamine (MBPE) were synthesized in our laboratory as described previously [22] and controlled for chemical and radiochemical purity before use. Unless specified in the text, all the experiments were performed at 4 °C.

Buffers

Buffer A (homogenization buffer) consisted of 50 mM Tris/HCl, pH 7.4, 250 mM sucrose, 1.5 mM EDTA and 0.01 % $\text{Na}_2\text{S}_2\text{O}_3$. Buffer B was buffer A without sucrose. Buffer C (dialysis buffer) contained 50 mM Tris/HCl, pH 6.8, 8 % (v/v) glycerol, 0.1 % SDS and 12 mM thioglycerol. Buffer D (elution gel buffer) contained 50 mM Tris/HCl, pH 7.9, 0.15 M NaCl, 0.1 mM EDTA, 12 mM monothioglycerol and 0.1 % SDS. Buffer E (chromatofocusing starting buffer) consisted of 25 mM Bistris/iminodiacetic acid, pH 7.1, 1 % (w/v) taurine and 1 % (v/v) Tween 20. Buffer F (chromatofocusing elution buffer) comprised 10 % (v/v) polybuffer 74/iminodiacetic acid, pH 4.1, 1 % (w/v) taurine and 1 % (v/v) Tween 20. Buffer G (enzymic assay buffer) contained 125 mM Tris/HCl, pH 9, and 0.1 mM EDTA.

Preparation of rat liver microsomes

Sprague–Dawley rats were ovariectomized by the dorsal route at least 3 weeks before being killed by cervical dislocation. The preparation procedure has been described in detail previously [23]. Briefly, livers were perfused with isotonic buffer and then homogenized in buffer A (2.5 vol./g of liver) with a Polytron B homogenizer. Protein concentrations were determined by the method of Bradford [24] with BSA as a standard.

Binding assays

Binding assays were routinely performed with [^3H]tamoxifen [6] and [^3H]azido-MBPE [22]. Assays were performed in buffer B for 18 h at 4 °C in a volume of 200 μl with 20 μg of membranous proteins and were incubated with various concentrations of radioligand including 1 μM oestradiol to mask tamoxifen binding to residual ER. Non-specific binding was determined in the presence of 2.5 μM tamoxifen. After incubation (18 h at 4 °C), bound and free radioligands were separated by filtration through a Sephadex LH-20 column [25] and the radioactivity was measured by liquid-scintillation counting. Scatchard analyses were performed with the LIGAND program [26].

Photoaffinity labelling

Rat liver microsomal proteins (250 mg) were adjusted to a protein concentration of 5 mg/ml in buffer B. The protein suspension was incubated overnight with 100 nM [^3H]azido-MBPE at 4 °C in the dark. The suspension was maintained under nitrogen flux and constant agitation. The mixture was then subjected to UV irradiation at 302 nm (exposure to four 15 W sources) for 20 min, 7 cm from the lights, under a Pyrex glass plate to filter out wavelengths below 300 nm. The mixture was then filtered through Whatman GF/A glass fibre filters. After an extensive washing with buffer B, the filters were extracted with buffer E for 2 h.

Chromatofocusing

Extracted samples were loaded on a 150 ml column of PBE 94 resin (Pharmacia Biotechnology) equilibrated in the same buffer.

Elution (30 ml/h) was performed with buffer F for 48 h; 10 ml fractions were collected. Ionic strength and pH were monitored with a mixed pH meter/conductimeter from LKB. A 1 ml aliquot was taken for radioactivity counting. A 50 ml fraction corresponding to pH 6.4 was concentrated 10-fold with a Centrprep 30 (Centricon) and then subjected to dialysis overnight (molecular mass cutoff 12–14 kDa) against buffer. Samples were freeze-dried, resuspended in buffer D and separated by SDS/PAGE [10 % (w/v) gel]. After electrophoresis the gels were stained with 0.2 % Coomassie Brilliant Blue in 20 % (v/v) methanol/0.2 % (v/v) acetic acid for 20 min without fixing, then destained in 30 % (v/v) methanol until bands became visible. Stained protein bands were digested in-gel with pig trypsin by the method of Rosenfeld [27]. The peptides eluted from the polyacrylamide matrix were purified by HPLC with a Brownlee Labs micro-gradient system on a C_{18} Ultrasphere column (250 mm $\times$ 2 mm). Elution was performed with a linear 1–60 % (v/v) gradient of acetonitrile in 0.1 % trifluoroacetic acid at a flow rate of 0.3 ml/min. The peaks were detected at 218 nm and the collection was manual. The N-terminal sequence analysis was performed on a gas-phase sequencer (470A, Applied Biosystems). The molecular masses of the peptides were measured with a laser mass spectrometer (Finnigan MAT). Protein sequence database searches were performed via the World Wide Web, with mass spectrometric data and PEPTISEARCH software [28] at the European Molecular Biological Laboratory, and by using Blast-Search at the National Biotechnology Information Center.

Inhibition of protein synthesis

A series of experiments was first conducted with cycloheximide (Sigma), an inhibitor of protein synthesis, to measure the half-life of AEBS in SKHep-1 cells. Specific protein inhibition was then performed with an oligonucleotide anti-sense strategy. As several studies have reported the inhibitory efficiency of 18-mer oligonucleotides matching the first 5' translated codons of mRNA encoding proteins [29], we chose the DNA sequence: 3'-TACA-CCGATCTTTAGGAG-5', corresponding to the anti-sense sequence of the first 18 bases of the open reading frame of human microsomal epoxide hydrolase (mEH). Controls were made with scrambled bases contained in the anti-sense oligodeoxynucleotide and with an inverted 5'/3' base sequence of the DNA, 3'-GAGGATTCTAGCCACAT-5'. The transfections of SKHep-1 cells with the oligonucleotides were performed with lipofectin in accordance with the manufacturer's recommendations (Gibco-BRL). Cells were harvested by scraping, 48 h after transfection, and sonicated in buffer A. mEH production was assessed by Western blotting after SDS/PAGE with a specific rabbit polyclonal antibody [30] kindly provided by Dr. P. H. Beaune. The peroxidase-conjugated anti-(rabbit Ig) (Dako) activity was revealed by the chemiluminescent detection system (Amersham); immunoquantification was performed by densitometry. AEBS binding parameters were measured as described previously [23].

Assay for enzymic mEH activity

The enzymic mEH activity was measured with styrene oxide, essentially as described by Seidegard et al. [31]. Rat liver microsomes were obtained as described previously [25] and were resuspended in buffer G containing 1.5 mM styrene oxide as mEH substrate. The reaction was initiated by the addition of microsomal proteins (1.2 mg) in 400 μl total volume, and proceeded at 4 °C. All the reactions were run in triplicate and were stopped by the addition of ethyl acetate (2 ml) in ice. The mixture was vortex-mixed vigorously and then centrifuged for 15 min at

1500 g. The organic layer was recovered and evaporated to dryness in a Savant Speed-Vac centrifuge. The products were solubilized in methanol and subjected to HPLC on a C₁₈ ODS Hypersyl column (250 mm × 4.6 mm; Shandon). Elution was performed with a linear 20–80% (v/v) gradient of methanol. Styrene glycol detection was monitored with UV at 254 nm.

Assay for binding of styrene oxide on AEBS

Scatchard analyses were performed as described above with rat liver microsomes to test the binding potency of styrene oxide on AEBS. Proteins (60 µg) were incubated with increasing concentrations of [³H]tamoxifen with or without 0.25 mM styrene oxide.

Human mEH cDNA open reading frame subcloning

Total RNA from the hepatocarcinoma cell line SKHep-1 was obtained by a rapid guanidinium thiocyanate procedure [32]. The cDNA encoding for mEH was obtained by reverse transcriptase-mediated PCR from total RNA by using the superscript preamplification system (Gibco-BRL) with random hexamers for the reverse transcription step. For PCR, oligonucleotides with *Eco*RI and *Bam*HI restriction sites were used, matching the first 15 and last 16 bases respectively of the open reading frame cDNA encoding the human mEH [33]. The amplification product was cloned in the *Eco*RI, *Bam*HI restriction sites of pSG5 vector [34] and sequenced by the dideoxy chain-termination technique [35].

Overproduction of the epoxide hydrolase protein

Plasmid (10 µg) containing mEH was transiently expressed into the COS-7 cells by a DEAE-dextran transfection method [36]. Cells were harvested by scraping, 48 h after transfection, and sonicated in buffer A. mEH production was assessed by Western blotting after SDS/PAGE with a specific rabbit polyclonal antibody [30]. The peroxidase-conjugated anti-(rabbit Ig) (Dako) activity was revealed by the chemiluminescent detection system (Amersham). AEBS binding parameters were measured as described previously [23].

RESULTS

Purification of the photolabelled proteins

The microsomal proteins were photolabelled with [³H]azido-MBPE in the presence or absence of tamoxifen (2.5 µM) then solubilized in buffer F and subjected to chromatofocusing. An aliquot of each fraction of the flow-through was counted for radioactivity. Radioactive peaks were collected and dialysed overnight against buffer G, concentrated by ultrafiltration with Centrprep 30 (Amicon) and resuspended in buffer D. Microsomal proteins (100 ng) (Figure 1A, lane a) and purified proteins (100 ng) (Figure 1A, lane b) were subjected to SDS/PAGE [8% (w/v) gel]. The gel was coloured with silver (Figure 1A). Purification gave two proteins with mobilities corresponding to molecular masses of 50 and 40 kDa (Figure 1A, lane b). Analysis of the radioactivity incorporated after photoaffinity labelling of microsomal (Figure 1B, ●) or purified (Figure 1B, ○) proteins after SDS/PAGE showed that two peaks present in crude microsomes were also present after purification with mobilities corresponding to molecular masses of 50 and 40 kDa (Figure 1B). Photolabelling was abolished in the presence of tamoxifen (results not shown). This showed that the photoaffinity-labelled proteins from the microsomes had been co-purified, and

Figure 1 Purification of AEBS

Microsomal proteins (250 mg) were purified after photoaffinity labelling with [³H]azido-MBPE and detergent solubilization by chromatofocusing through a mono-P column as described in the Materials and methods section. (A) Crude microsomal protein (100 ng) (lane a) or purified proteins (100 ng) (lane b) were separated by SDS/PAGE [10% (w/v) gel] and stained with silver. (B) Photoaffinity labelling of proteins was measured after separation of 50 µg of microsomal proteins (●) and 100 ng of purified proteins (○) by SDS/PAGE [8% (w/v) gel]. Gels were sliced into 2 mm pieces and crushed, after which the radioactivity was counted. Gels were calibrated with molecular mass standards: rabbit skeletal muscle myosin (200 kDa), β-galactosidase from *Escherichia coli* (116.2 kDa), rabbit muscle phosphorylase B (97.4 kDa), BSA (66.2 kDa), hen egg white ovalbumin (45 kDa) and bovine carbonic anhydrase (31 kDa).

suggested that these proteins might be associated in a functional complex in the intact membranes and that AEBS might be a hetero-oligomeric complex containing them.

In-gel digestion of proteins and internal peptide sequences

The 50 kDa protein was proteolysed in the gel. Peptides eluted from the gel were separated by microbore reverse-phase HPLC. The partial sequence of four peptides is given in Table 1. These sequences were contained in the primary sequence of the microsomal rat epoxide hydrolase (EC 3.3.1.1) (peptide 20, amino acid sequence 352–410; peptide 21, amino acid sequence 22–34; peptide 23, amino acid sequence 245–254; peptide 25, amino acid sequence 256–266) [37]. We were unable to obtain microsequences from peptides generated by trypsinolysis of the 40 kDa protein. Mass spectrometric analysis, however, gave masses for four

Table 1 Partial amino acid sequences of tryptic peptides from purified AEBS

The purified AEBSs were separated by SDS/PAGE [10% (w/v) gel] and stained with Coomassie Blue. The 50 kDa protein was proteolysed in the gel with trypsin, and peptides were separated by microbore reverse-phase HPLC as described in the Materials and methods section. Amino acid sequences are given with the one-letter codes.

Peptide no.	Sequence
20	V F V P T G F S A F P S E L L H A P E
21	D K E E T L P L G D G W W
23	G L H L N M A F I S
25	S F Y T M T P L L G Q

Figure 2 Western blot analysis of mEH protein and Scatchard plot of [³H]tamoxifen binding after treatment of SKHep-1 cells with anti-sense mEH

(A) Microsomal proteins (50 µg per lane) from SKHep-1 cells were subjected to SDS/PAGE [10% (w/v) gel]. After transfer to nitrocellulose sheets, bands were revealed with anti-mEH polyclonal antibody. Lane 1, control cells; lane 2, inverted oligonucleotides; lane 3, anti-sense oligonucleotides; lane 4, scrambled oligonucleotides. The gel was calibrated with molecular mass standards as listed in the legend to Figure 1. (B) Scatchard plot of tamoxifen binding on microsomal proteins from control cells (○), inverted oligonucleotides (*), anti-sense oligonucleotides (△) and scrambled oligonucleotides (▽).

peptides. These masses (*m/z*) were 1325, 1464, 1476 and 1508, and did not correspond to the tryptic peptides of mEH or of any known protein from the rat liver currently listed in protein

Figure 3 Effect of tamoxifen and MBPE on mEH catalytic activity

Inhibition of mEH activity by tamoxifen (●) and MBPE (□). Inhibition is expressed as the percentage of residual styrene oxide hydrolase activity plotted against the logarithm of the concentration of inhibitor. Points are means $\pm$ S.D. for duplicate assays from three experiments. Under the conditions described in the Materials and methods section the IC_{50} was 19.22 µM for tamoxifen and 10.58 µM for MBPE. IC_{50} values were calculated with Graphpad PRISM software, version 2.1. The calculation of IC_{50} values was performed with data from triplicate assay tubes at each drug concentration. Eight different concentrations were tested for each inhibitor.

databases, including cytochromes *P*-450 and flavin mono-oxygenases.

Inhibition of mEH synthesis in hepatocytes

A series of experiments was conducted with anti-sense oligonucleotides able to match with the corresponding mEH mRNA to improve the involvement of the mEH in AEBS.

First a half-life time (mean $\pm$ S.D.) of 26 ± 2 h was measured for AEBS at saturation binding (15 nM [³H]tamoxifen) when SKHep-1 cells were treated with cycloheximide (results not shown). This enabled us to select an optimal time for treatment of these cells with anti-sense oligodeoxynucleotides. A period of 48 h might give 70 % inhibition of mEH neosynthesis.

Lipofectin was chosen for transfection, and primary experiments were performed to determine the most efficient DNA-to-lipofectin ratio and time of treatment to inhibit mEH without any cytotoxic effect on SKHep-1 cells. As expected, 48 h of treatment with 20 µM anti-sense oligonucleotides was able to inhibit epoxide hydrolase synthesis (Figure 2A) and concomitantly the AEBS content was strongly diminished (Figure 2B). A Scatchard representation of [³H]tamoxifen binding exhibited a B_{max} of 3.3 ± 0.2 pmol/mg of protein for control cells compared with 0.82 ± 0.3 pmol/mg of protein for anti-sense-treated cells (means $\pm$ S.D.), indicating a 75 % decrease in mEH expression. The affinity for tamoxifen was not significantly affected. Controls performed with non-specific oligonucleotides (scrambled and 5'/3' inverted oligonucleotide) had no effect either on mEH neosynthesis (Figure 2A) or on [³H]tamoxifen binding (Figure 2B).

Regulation of the enzymic activity of mEH

Studies *in vitro* were performed on crude rat liver microsomal proteins and the production of styrene glycol was estimated by HPLC as described in the Materials and methods section. Kinetic studies were performed at 4 °C. Styrene glycol production was linear up to 30 min and then reached a plateau. The mEH activity was determined by measuring the slope of the curve for each treatment. As shown in Figure 3, both tamoxifen and

Figure 4 Scatchard plots of [^3H]tamoxifen binding to microsomal AEBS without or with styrene oxide

Rat liver microsomal proteins (20 μg per assay) were incubated with increasing concentrations of [^3H]tamoxifen (0.3–30 nM) in the absence (○) or presence (●) of 0.25 mM styrene oxide. Experiments were performed as described in the Materials and methods section. Each value is the mean of triplicate determinations.

Figure 5 Overexpression of mEH

Left panel: Scatchard plot of [^3H]tamoxifen binding in COS-7 cells. Symbols: ○, pSG-5; ●, pSG-5 mEH plasmid. Right panel: Western blot analysis of COS-7 cells transfected with the pSG-5 or pSG-5 mEH plasmid; 50 μg of cell proteins was subjected to SDS/PAGE [10% (w/v) gel]. After transfer to nitrocellulose sheets, bands were revealed with anti-mEH polyclonal antibody as described in the Materials and methods section. Results are representative of three individual experiments.

MBPE inhibited mEH in a dose-dependent manner. The calculated IC_{50} values were 19.22 and 10.58 μM respectively.

Binding of styrene oxide on AEBS

Scatchard analyses were performed for [^3H]tamoxifen in rat liver microsomes in the presence or absence of a concentration of styrene oxide at the K_m for mEH. Figure 4 shows that [^3H]tamoxifen bound AEBS with a K_d of 3.6 ± 0.2 nM and a B_{max} of 36.7 ± 0.4 pmol/mg of protein. In the presence of styrene oxide K_d was 5.1 ± 0.3 nM and B_{max} was 37.1 ± 0.3 pmol/mg of protein. Styrene oxide produced a diminution of to 1/1.4 in the affinity of tamoxifen for AEBS without affecting the B_{max} . This shows that styrene oxide might be a competitive inhibitor of tamoxifen on AEBS.

Overexpression of mEH

The open reading frame of the mEH cDNA was subcloned into the pSG5 vector and transiently expressed into the COS-7 cells. Overexpression of mEH was analysed by Western blotting (Figure 5) and showed one band at 50 kDa. COS-7 cells, however, displayed a basal cross-immunoreactivity with anti-mEH antibody, showing that endogenous mEHs were constitutively expressed by COS-7 cells (results not shown). Scatchard analyses did not indicate a significant increase in binding of [^3H]tamoxifen (Figure 5) or [^3H]azido-MBPE (results not shown). As expected, this indicates that mEH is not sufficient to reconstitute the binding activity of tamoxifen.

DISCUSSION

Photolabelling experiments of the particulate fraction of rat liver microsomes with [^3H]azido-MBPE and separation of the proteins in denaturing conditions by SDS/PAGE gave two labelled proteins of molecular masses 40 and 50 kDa (Figure 1B), as shown previously [22]. A three-step purification procedure with the use of chromatofocusing, extensive dialysis and SDS/PAGE [10% (w/v) gel] yielded two proteins of molecular masses 40 and 50 kDa coloured with silver (Figure 1A). The radioactivity profiles associated with the proteins separated by SDS/PAGE were qualitatively the same before and after purification (Figure 1B), which meant that we had co-purified the two photolabelled proteins. This showed that they were associated proteins and that both might be subunits of AEBS. This is consistent with mass assessment experiments under non-denaturing conditions that gave a molecular mass of 100 kDa for the tamoxifen-binding site [6].

Purified proteins were proteolysed into the polyacrylamide to produce peptides suitable for sequencing or mass determination. After purification the tryptic peptides were sequenced by the Edman method. The primary sequences of the peptides generated from the 50 kDa protein corresponded to the sequence of the mEH. Although the 40 kDa protein was detectable at the same level as the 50 kDa on the basis of densitometric analysis after colouring with silver, we obtained one-third to one-fifth the number of tryptic peptides compared with the 50 kDa protein, which was not enough for sequence analysis. This might be due to either a lower efficiency of trypsinolysis because of higher hydrophobicity of the 40 kDa protein, or a lower solvent extractability of the tryptic peptides. However, although at least 1–5 pmol of peptide are required for N-terminal sequencing, subpicomole levels of peptide are useful for mass spectrometric approaches owing to their protein identification sensitivity [38]. Database searching did not give matching results with any protein known at present. This excluded the possibility that the 40 kDa protein might be a product of degradation of mEH (the 50 kDa protein) or proteins known to be potentially associated with mEH, such as cytochrome *P*-450 or flavin mono-oxygenase.

To examine whether the protein identified as mEH was related to AEBS, we first inhibited the synthesis of mEH in hepatocytes by using anti-sense technology. The number of AEBSs was reduced by two-thirds and was associated with a diminution of mEH expression as detected by Western blot analysis. This is consistent with the expected residual mEH activity of 30% after 48 h. This showed that mEH expression is related to tamoxifen-binding activity.

Moreover tamoxifen and MBPE are inhibitors of the catalytic activity of mEH *in vitro*. Conversely the mEH substrate styrene oxide is a competitive inhibitor of tamoxifen binding to AEBS. This clearly demonstrates the relationship between AEBS and mEH. The affinities of tamoxifen and MBPE for AEBS are in the

nanomolar range, which is four orders of magnitude smaller than the IC_{50} measured in the hydrolysis of styrene oxide, which is a weak substrate for mEH. Substrates or inhibitors of mEH are of variable structure but some series contain aromatic moieties such as stilbene, diphenylmethane, benzophenone and chalcone [39,40] that are found in the chemical structure of AEBS ligands [41–43]. The aromatic moiety of AEBS ligand might affect the catalytic activity of mEH.

Overexpression of mEH in COS-7 cells did not significantly modify the binding parameters of tamoxifen. The expression of mEH was monitored by Western blot analysis and displayed a high expression level in transfected cells compared with untransfected cells. This showed that mEH alone was not the binding site of tamoxifen and that one or more additional structures might be required to bind it. One such factor might be the protein of molecular mass 40 kDa that has not yet been identified. Taken together, these results provide evidence that mEH is necessary but not sufficient to reconstitute AEBS (without increasing the binding of AEBS ligands). Moreover the heterologous expression of mEH in host cells that expressed endogenous AEBS could signify that full binding activity might require a stoichiometric quantity of cofactors.

Epoxide hydrolases are well known as type II enzymes involved in the detoxification of xenobiotics. Their function is to catalyse the hydrolysis of a wide variety of aliphatic and aromatic electrophilic epoxides to vicinal diols. This activity might be related in some way to tamoxifen toxicity or genotoxicity. Tamoxifen has been shown to induce DNA and protein adducts in rat tissues [44,45], and a correlation between DNA adduct formation and carcinogenicity has been established [46]. Tamoxifen has been shown to be metabolized into epoxide derivatives [47,48] that are potent substrates for mEH and might be responsible for DNA and protein adducts. We have shown that tamoxifen is an inhibitor of mEH and that it might block the catalytic hydrolysis of its own epoxy metabolites or other epoxy metabolites to allow them to produce DNA and protein adducts. These observations might explain the relationship between the binding to a subunit of AEBS and tamoxifen genotoxicity, but the relationship between the growth control of tamoxifen and AEBS ligands remains to be explored.

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