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HIGH AFFINITY SPECIFIC ANTIESTROGEN BINDING SITES ARE CONCENTRATED IN ROUGH MICROSOMAL MEMBRANES OF RAT LIVER

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Saturation and competitive binding analyses demonstrated the presence of a high affinity (K_D = 0.92 nM), specific antiestrogen binding site (AEBS) in rat liver microsomes and at least 75% of total liver AEBS was recovered in this fraction. When microsomes were further separated into smooth and rough fractions, AEBS was concentrated in the latter. Subsequent dissociation of ribosomes from the rough membranes revealed that AEBS was associated with the membrane and not the ribosomal fraction. Antiestrogen binding activity could not be extracted from membranes with 1 M KCl or 0.5 M acetic acid but could be solubilized with sodium cholate. These data indicate that AEBS is an integral membrane component of the rough microsomal fraction of rat liver.

The synthetic nonsteroidal antiestrogens are known to interact with the estrogen receptor (ER) system of estrogen target cells (1). More recently they have also been shown to bind to another high affinity intracellular binding site (the antiestrogen binding site, AEBS) which does not bind steroid hormones but has a high affinity for antiestrogenic compounds of the triphenylethylene series (2-10). Following the initial description of AEBS in cytosol from chick oviduct (2) and a number of other estrogen target tissues (3) some groups were unable to confirm these observations with rat uterus (11) and MCF 7 cell cytosol (12). Since a possible explanation for these discrepancies was that AEBS was associated predominantly with a particulate fraction rather than cytosol we conducted a series of experiments in which rat liver was fractionated and the subcellular location of this binding site defined. These novel data which demonstrate that AEBS is an integral membrane protein component of rough microsomes should facilitate further studies on the characterization and functional definition of this molecule.

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MATERIALS AND METHODS

Materials. [^{3}H]tamoxifen ([^{3}H]TAM), clomiphene, tamoxifen and their analogs were supplied and stored as previously reported (6,10). All aqueous solutions were prepared in 10 mM Tris-HCl, 25 mM KCl buffer pH 7.4 at 22°C (TK) containing 2 mg/ml bovine serum albumin (BSA; Calbiochem-Behring Corp., La Jolla, CA).

Subcellular Fractionation. Livers were removed from 18 hr starved female Sprague-Dawley or Fischer rats (200 - 250 gms) and rapidly cooled on ice. The tissue was minced, washed in ice cold TK containing 0.25 M sucrose (TSK) and homogenized (20% w/v in TSK) in a Teflon-glass Potter-S homogenizer (6 strokes at 1000 rpm). The homogenate was filtered through 100 μ nylon mesh. Centrifugation at 800 x g for 10 mins gave the crude nuclear pellet (N) which was washed twice with TSK. The combined supernatants were centrifuged at 10,000 x g for 20 mins to produce the mitochondrial pellet (M) which was washed twice with TSK. The combined supernatants from this fraction were further centrifuged at 130,000 x g for 1 hr to produce the microsomal fraction (Mc), which was washed with TSK containing 150 mM KCl. The combined supernatants from the latter fraction constituted the cytosol (C). All pellets were resuspended in TSK. Rough (RM) and smooth microsomes (SM) were prepared by the method of Dallner (13). RM which had been stored at -20°C for < 24 hr were used to prepare free ribosomes and stripped membrane fractions by treatment with puromycin/KCl (14) or EDTA (13).

To test extraction of AEBS from microsomes, solutions of KCl, acetic acid or detergent were added to microsomal suspensions. After 30 mins at 0°C the preparations were centrifuged at 130,000 x g for 1 hr and the supernatant and resuspended pellets assayed for binding activity.

Enzyme Assays. 5'nucleotidase (EC 3.1.3.5) was assayed by the method of Newby et al. (15). Cytochrome c oxidase (EC 1.9.3.1.), NADPH cytochrome c reductase (EC 1.6.2.4) and β-glucuronidase (EC 3.2.1.31) were assayed as described by Beaufay et al. (16) while lactate dehydrogenase (EC 1.1.1.27) was assayed by the method of Schwartz and Bodansky (17). RNA was measured by a modified Schmidt-Thanhauser method as described by Munro and Fleck (18). Protein concentration was determined by the method of Lowry et al. (19) using BSA as a standard. Results of the subcellular fractionation studies are presented according to the method of de Duve et al. (20).

Binding of TAM to AEBS. All binding assays were done in the presence of 1 μM estradiol to eliminate interaction of [^{3}H]TAM with the estrogen receptor. Saturation and competitive binding analyses were performed as previously described (6,10). In the subcellular fractionation studies, incubations of the fractions with [^{3}H]TAM were charcoal-separated by centrifugation at 1500 x g for 10 min. To detect the presence of any labelled material co-pelleting with charcoal-dextran, a parallel set of incubations was treated with 0.5 ml TK-BSA (2 mg/ml) for 30 min at 4°C and centrifuged as above. The pellets were washed twice with TK-BSA dissolved in 0.7 ml Protosol (New England Nuclear): ethanol (1:1) and 0.5 ml aliquots containing 25 μl glacial acetic acid were counted. The sum of the binding in the charcoaled supernatant and in the pelleted material was thus equal to the total binding within a particular incubation.

RESULTS

When the binding of [^{3}H]TAM to the microsomal fraction and the RM and SM sub-fractions from rat liver was analyzed according to the Scatchard transformation (Fig. 1A) a curvilinear relationship between [B]/[U] and [B] was apparent. However, following correction for non-specific binding the relationship was linear, demonstrating the presence of a single class of

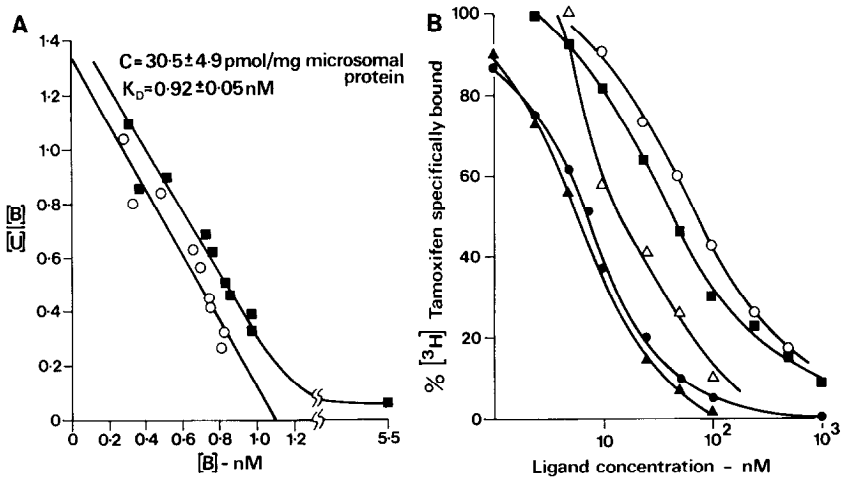

Fig. 1. BINDING OF ANTIESTROGENS TO RAT LIVER MICROSOMES.
A. Scatchard plot of the interactions between [^{3}H]TAM and the AEBS in rat liver microsomes. Data are plotted before (■) and after (○) correction for non-specific binding.
B. Competition of antiestrogens for [^{3}H]TAM binding to AEBS in rat liver microsomes. Tamoxifen (●), N-desmethyltamoxifen (○), 4-hydroxytamoxifen (■), clomiphene (▲), 9599 (△). The relative binding affinities were tamoxifen = 100, N-desmethyltamoxifen = 11, 4-hydroxytamoxifen = 17, clomiphene = 136, 9599 = 46.

non-interacting, high affinity saturable binding sites. The K_D of this interaction, which was not significantly different in the above three fractions, was 0.92 ± 0.05 nM (mean ± S.E.M., n = 22). The AEBS concentration in the microsomal fraction was 30.5 ± 4.9 pmol/mg protein or 0.78 ± 0.10 nmol/g liver.

Preliminary studies on the specificity of the rat liver microsomal AEBS demonstrated a lack of affinity for sex steroid hormones (data not shown) and high affinity for analogs of the nonsteroidal antiestrogens clomiphene and tamoxifen (Fig. 1B). As has been previously reported with AEBS from MCF 7 human mammary carcinoma cells (3,6,10) modifications in the alkyl amino ether side chain influence affinity for AEBS. Thus removal of a methyl group from tamoxifen or an ethyl group from enclomiphene to form the secondary amines, N-desmethyltamoxifen and 9599, respectively, markedly reduced affinity (Fig. 1B). Aromatic hydroxylation of tamoxifen to 4-hydroxytamoxifen also resulted in a marked reduction in affinity for liver AEBS.

Figure 2 shows the distribution of AEBS and the sub-cellular organelle enzyme markers between the cytosol, mitochondrial, microsomal and nuclear

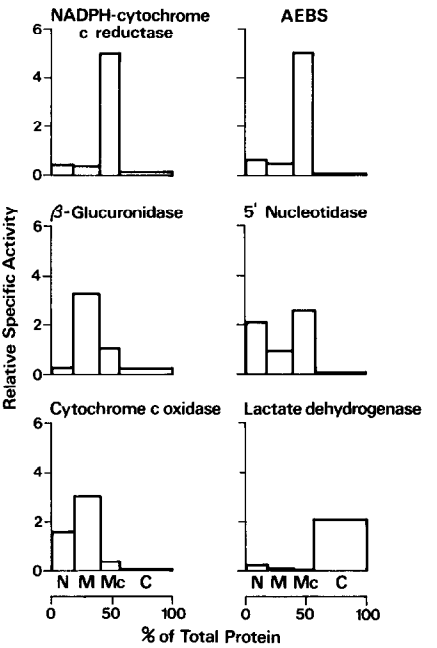

Fig. 2. SUBCELLULAR LOCALIZATION OF AEBS IN RAT LIVER.
Rat liver homogenates were separated by differential centrifugation into nuclear (N), mitochondrial (M), microsomal (Mc) and cytosol(C) fractions as described in "Materials and Methods". The distribution of marker enzymes for plasma membranes (5'nucleotidase), endoplasmic reticulum (NADPH cytochrome c reductase), lysosomes (β-glucuronidase), mitochondria (cytochrome c oxidase) and cytosol (lactate dehydrogenase) and the distribution of AEBS in each fraction are shown.

fractions of rat liver. The AEBS was concentrated in the microsomal fraction and throughout the fractions paralleled closely the distribution of the endoplasmic reticulum marker enzyme, NADPH cytochrome c reductase. The microsomal fraction contained 78.4 ± 2.1% of the total recovered AEBS. The cytosol had < 1% of the detectable AEBS but appreciable quantities were detected in crude nuclear and mitochondrial fractions (Fig. 2). This was probably due to contamination by microsomal components, as evidenced by similar proportions of NADPH cytochrome c reductase in these fractions.

Figure 3 presents the distribution of AEBS between the rough and smooth microsomal subfractions. The concentration of AEBS in the RM fraction and the similarity with the distribution of RNA suggests an association of AEBS with rough endoplasmic reticulum. The specific activities of AEBS, RNA and NADPH cytochrome c reductase in SM (expressed as a % of that in the RM) were 62.1 ±

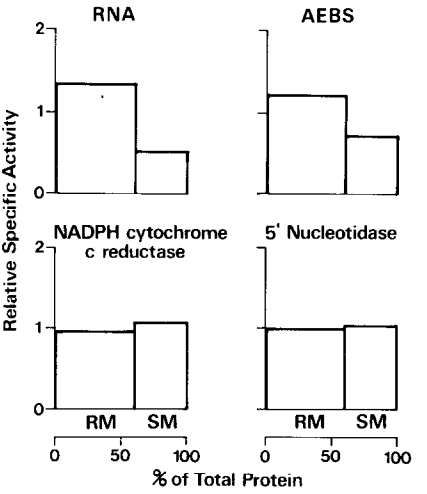

Fig. 3. DISTRIBUTION OF AEBS BETWEEN SUBFRACTIONS OF RAT LIVER MICROSOMES. Microsomes were separated on a discontinuous sucrose gradient containing CsCl into RM and SM fractions as described in "Materials and Methods". The distribution of marker enzymes for plasma membranes, endoplasmic reticulum and the distribution of AEBS and RNA are shown.

4.8, 39.9 ± 4.9 and 143 ± 19 respectively. When RM were stripped of bound ribosomes and separated into stripped membrane and ribosomal fractions AEBS was found only in the membrane fraction but with a 47 ± 8% reduction in specific activity compared with the starting material.

AEBS activity was not significantly extracted from microsomes by 0.15, 0.5 or 1 M KCl or by 0.5 M acetic acid. However sodium cholate was able to partially solubilize AEBS, using the criterion of lack of sedimentation at 130,000 x g for 1 hr. At a microsomal protein concentration of 3 mg/ml, sodium cholate at concentrations of 0.3, 1 and 2% (w/v) solubilized 5, 20 and 45% of the AEBS, respectively. Sodium deoxycholate also solubilized AEBS but with low yield. Other detergents tested (CHAPS, CHAPSO, digitonin, taurodeoxycholic acid and β-D-octyl glucoside) failed to achieve solubilization and/or destroyed binding activity.

DISCUSSION

This study confirms the presence of a specific, high affinity binding site for nonsteroidal antiestrogens in rat liver but demonstrates that this site is located almost exclusively in the microsomal fraction and not the cytosol as

previously suggested (3,8). Less than 1% of total liver AEBS was present in the cytosol while > 60% of the activity associated with crude nuclei was removed following purification through 2.2 M sucrose (23). It is still unclear whether this represents inadequate purification or a distinct nuclear binding site for TAM. Such results, however, bring into question the validity of recent hormonal regulation studies on AEBS where inadequate subcellular separation techniques were employed or only a portion of the total cellular AEBS was measured (8,21).

Competition studies with a limited range of ligands indicated that the rat liver microsomal AEBS had similar specificity to AEBS extracted from other tissues (2-10). Indeed 4 of the 5 compounds presented in Fig. 1B showed identical relative binding affinities to those previously reported for MCF 7 cell microsomal AEBS (10). 4-hydroxytamoxifen was at least 4-fold less potent in liver cf. breast cells and this may be due to binding of this ligand to other components within the rat liver microsomes.

Although it has recently been suggested that AEBS is predominantly microsomal in rat uterus (7) and human mammary carcinoma cells (9,10) this is the first demonstration that AEBS is an integral component of rough microsomal membranes which are presumed to be rough endoplasmic reticulum. The failure of 1 M KCl or 0.5 M acetic acid to solubilize AEBS suggests that it is not a loosely adsorbed or extrinsic membrane protein. Furthermore the effective solubilization of AEBS only at relatively high concentrations of sodium cholate suggest that AEBS is not a luminal protein of the microsomal vesicles. The reduction in specific activity of AEBS following removal of ribosomes from rough membranes may indicate that their removal renders AEBS more susceptible to degradation or that the presence of ribosomes is essential for binding site integrity.

This paper has demonstrated that rat liver AEBS has properties similar to AEBS from other sources but is present at much higher concentrations (2-10). The demonstration of the true subcellular localization of AEBS and presentation of strategies to solubilize it should facilitate further investigations into the biochemistry and physiological significance of this interesting binding protein.

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