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26. Antiestrogens

INTERACTIONS OF TAMOXIFEN IN THE CHICKEN

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Summary—The triphenylethylene antiestrogens are very potent antagonists of estrogen action in the chicken and manifest little agonist activity compared to their action in other species. The estrogen antagonism is most probably mediated by the estrogen receptor, to which tamoxifen binds with a K_i of 2.6 nM. Tamoxifen is readily metabolized by liver to 4-hydroxytamoxifen, which binds the liver nuclear estrogen receptor with a K_i of 0.1 nM. The K_d of the receptor is 0.7 nM. Estrogen receptor concentrations in liver from immature chickens are relatively low both in nuclear and cytosol fractions. Treatment with estradiol results in 10-fold up-regulation of the nuclear levels to give a total receptor concentration of about 2 pmol/g tissue. Tamoxifen can promote this up-regulation to a limited extent, but interpretation of experimental results is compromised by difficulties with exchange assays in the face of the very high binding affinity of 4-hydroxytamoxifen.

Tamoxifen also binds with high affinity (K_d 2–4 nM) and distinctive specificity to antiestrogen binding sites (AEBS) present in a wide variety of chicken tissues and in the highest concentration in the liver (800 pmol/g tissue). Liver and serum contain ether-soluble components which can compete for binding of [^{3}H]tamoxifen to the AEBS. The serum AEBS inhibitory activity is chromatographically heterogeneous and is associated with a sterol-like fraction as well as with a fatty-acid-containing fraction.

Tamoxifen treatment of cockerels results in dose- and time-dependent decreases in serum free and esterified cholesterol, and in phospholipids and triglycerides. These changes may reflect estrogen-receptor-independent interactions of tamoxifen.

INTRODUCTION

The study of estrogen action in the chicken has occupied an important niche in modern molecular endocrinology. Estrogenic induction of egg white proteins in chicken oviduct and of egg yolk proteins in the liver provides an excellent system for the analysis of many basic aspects of the mechanism of steroid hormone regulation of specific gene expression [1–3]. Inevitably, the actions of the triphenylethylene antiestrogens have been explored in the chicken and the general findings have been that the drugs predominantly display estrogen antagonistic actions with very little agonistic activity [4–6]. This contrasts with the situation in the rat where the triphenylethylene antiestrogens generally act as partial agonists and in the mouse where they are mainly agonistic (reviewed in [7]). Such distinctive species specificity is particularly interesting and may be explained by differences in the structural properties of the drug–estrogen receptor complexes [8]. Comparative studies on the antiestrogens thus have the potential to yield valuable insights into basic mechanisms.

Several review articles summarizing studies on triphenylethylene antiestrogens in the chicken have been published [9–11]. The most recent of these [11] covers the published literature up to and including 1984. The present paper therefore will concentrate on selected topics and more recent findings from this laboratory.

HIGH AFFINITY BINDING OF TAMOXIFEN TO CHICKEN LIVER COMPONENTS

The estrogen receptor system in chicken liver is characterized by relatively low levels of receptor in both cytosol and nuclear fractions in immature birds (about 200 fmol/g tissue in either compartment) [12]. Injection of estradiol results in a dose- and time-dependent increase in the nuclear salt-extractable receptor (K_d 0.7 nM) to give a concentration of about 2.5 pmol/g liver by 48 h after treatment with a dose of 25 mg/kg body wt. The mechanism of the increase probably involves induction of synthesis or stabilization of the receptor [13, 14]. A good question is whether or not tamoxifen or a tamoxifen metabolite can induce this up-regulation of the estrogen receptor.

Tamoxifen, and its 4-hydroxylated metabolite, bind to the nuclear salt-soluble estrogen receptor with K_i values of 2.6 and 0.1 nM respectively [15]. As has been pointed out elsewhere, the particularly high affinity of 4-hydroxytamoxifen compared to estradiol itself has the potential to compromise interpretation of results of exchange assays for nuclear estrogen receptor in liver fractions from animals previously treated with tamoxifen [10, 15]. 4-Hydroxytamoxifen, occupying the receptor sites, may be difficult to dissociate during the usual assay conditions. Also, antiestrogens bound to non-receptor sites in the nuclear preparation may dissociate during the assay incubation and thus interfere with the receptor assay. Evidence that both of these phenomena can occur with chicken liver has been presented [15]. The net result is that it has been difficult to assess the efficacy of antiestrogens in up-regulating the nuclear estrogen receptor. Certainly a relatively low dose of tamoxifen or 4-hydroxytamoxifen (6 mg/kg) can promote a 5-fold increase in salt-soluble nuclear receptor (Table 1), but levels comparable to those obtained by treatment

Table 1. The effect of injection of antiestrogens on the apparent concentration of nuclear estrogen receptor in salt extracts and in intact nuclei*

Treatment (4 h)	Specific binding (fmol/μg DNA)	
	Salt extracts	Intact nuclei
Estradiol (3 mg/kg)	0.46 ± 0.07	0.40 ± 0.12
Untreated control	0.05 ± 0.04	0.07 ± 0.02
Tamoxifen (6 mg/kg)	0.27 ± 0.04	0.08 ± 0.02
4-Hydroxytamoxifen (6 mg/kg)	0.24 ± 0.02	0.07 ± 0.02

*Estradiol or antiestrogen was administered intramuscularly and liver nuclei and salt extracts were prepared and assayed for specific [^{3}H]estradiol binding activity. The salt extracts were charcoal-treated before assay by incubation with 1.7% charcoal suspension for 15 min at 37°C, which was demonstrated to permit exchange of 60–70% of bound 4-hydroxytamoxifen. Adapted from Ref. [15].

with an optimal dose of estradiol have not been found. Experiments using higher doses of tamoxifen or with nuclear matrix or intact nuclei have not been carried out under conditions in which exchangibility or the possibility of high endogenous unlabeled ligand concentrations interfering with the assay were considered [10, 16]. This point is illustrated in Table 1, which clearly demonstrates that receptor sites can be seen in nuclear preparations from antiestrogen-treated animals only if extraordinary steps are taken to ensure ligand exchange. Proof of whether or not tamoxifen can fully induce estrogen receptor up-regulation in liver will probably require assays for receptor protein using specific antibodies.

After [^{3}H]-labeled tamoxifen became available, it soon was obvious that the triphenylethylenes could bind to cellular components other than the estrogen receptor system. Sutherland and colleagues first demonstrated high-affinity antiestrogen-specific sites in a cytosol fraction from the chicken oviduct [17]. It later became apparent that the sites were predominantly concentrated in the microsomal fraction in various mammalian tissues, particularly liver [18, 19]. Some activity has also been shown both in salt-soluble [20] and -insoluble [21] fractions of purified nuclei. Our experience with the chicken liver system is that the 5000-***g*** supernatant fraction from liver prepared by homogenization in a glycerol-containing buffer contains the equivalent of 800–1000 pmol of AEBS activity/g liver [11]. Very little of this activity is sedimented by centrifugation at 33,000 ***g*** but a considerable proportion (>50%) is sedimented at 100,000 ***g***. The AEBS activity remaining in the supernatant fraction (≈40%) is probably still associated with membranes, because sucrose density gradient centrifugation of this fraction reveals specific [^{3}H]tamoxifen binding activity sedimenting with a coefficient varying from 10 to 30 S [11].

CHARACTERIZATION AND SOLUBILIZATION OF LIVER AEBS

Initial characterization of AEBS in cockerel liver cytosol was carried out on the 100,000-***g*** supernatant from liver homogenized in buffer containing 50% glycerol [22]. A particular advantage of using this fraction is that the fluid material can be stored at −20°C for several months without any loss of activity. In contrast, 100,000-***g*** pellet fractions resuspended in a Tris buffer are relatively unstable and cannot withstand freezing and thawing [11].

Figure 1 shows the specificity of [^{3}H]tamoxifen binding to the chicken liver cytosol prepared in the glycerol-containing buffer. The ligand specificity and binding affinity (K_d, 2–4 nM) agree well with our earlier findings for chick liver nuclear AEBS and with work of others on AEBS from different species [17, 18, 23, 24]. The relative binding affinities show distinctive AEBS-type specificity in the following order: nafoxidine (183%) > tamoxifen (100%) > N-desmethyltamoxifen (42%) > 4-hydroxytamoxifen (32%) ≫ diethylstilbestrol (<0.001%). In contrast, for the chicken liver nuclear estrogen

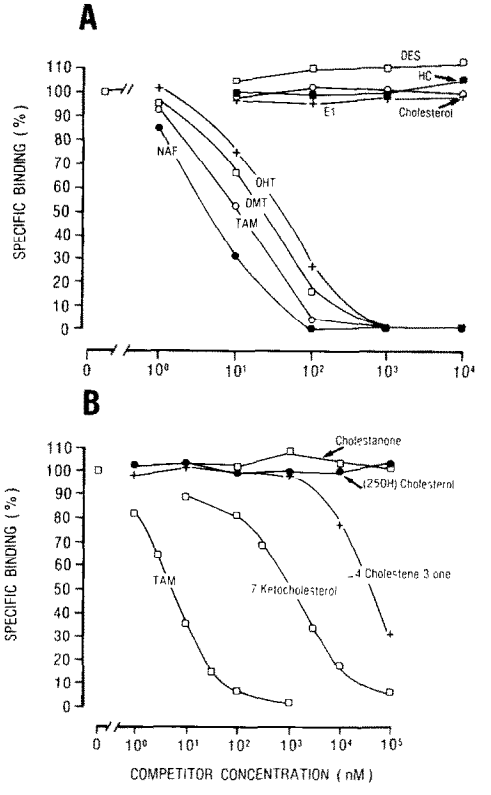

Fig. 1(A, B). Binding specificity of chicken liver cytosol AEBS. [^{3}H]Tamoxifen (10 nM) was incubated with the AEBS and varying concentrations of unlabeled competitor. NAF, Nafoxidine; TAM, tamoxifen; DMT, N-desmethyltamoxifen; OHT, 4-hydroxytamoxifen; E_1, estrone; HC, hydrocortisone. Adapted from Ref. [21] with permission.

receptor the following order of relative binding affinities is observed: 4-hydroxytamoxifen (400%) > estradiol (100%) > N-desmethyltamoxifen (28%) > tamoxifen (9%) [15]. Hydroxylation of tamoxifen at the 4 position thus decreases affinity for binding AEBS while increasing the affinity of estrogen receptor. Figure 1 also shows that estrone, cholesterol, hydrocortisone, cholestanone and 25-hydroxycholesterol have no demonstrable capacity to bind AEBS over a wide range of concentrations. Surprisingly, 7-ketocholesterol and Δ-4-cholestene-3 one display low but distinct and reproducible binding ability. These compounds also compete for [^{3}H]tamoxifen binding to the soluble nuclear-AEBS with similar relative binding affinity (Murphy and Lazier, unpublished results). The significance of the competition by 7-ketocholesterol is not clear and might certainly be queried. Given the relative binding affinity of about 1% that of tamoxifen, an effective competitive concentration of 7-keto-cholesterol for interaction with AEBS would need to be in the micromolar range.

Solubilization and purification of the AEBS are essential to further understanding of their role and to that end we have investigated a variety of conditions for solubilizing the chicken liver microsomal AEBS (Bapat and Lazier, unpublished observations). Optimal yield of soluble binding sites from a 100,000 ***g*** pellet was found using 4.0% CHAPS detergent in buffer. The solubilized AEBS has a specific activity of about 5–10 pmol/mg protein and a K_d for [^{3}H]tamoxifen of 1–2 nM. On centrifugation in a 10–34% sucrose density gradient the solubilized AEBS prebound to [^{3}H]tamoxifen sedimented with a coefficient of about 8 S. The peak of [^{3}H]tamoxifen binding was completely abolished by inclusion of a 100× excess of radioinert tamoxifen in the preincubation mixture. Gel filtration of the solubilized AEBS on Sepharose CL-6B yielded a Stokes' radius of 75–80 Å. Calculation of molecular weight using the Stokes' radius and the sedimentation coefficient [25] gives a value of 265,000 Da and a frictional ratio (f/f_0) of 1.72, indicating that the native AEBS is very asymmetrical molecule or complex. A caveat must be that the small amount of detergent (0.49%) included in the gel filtration elution buffer might affect the hydrodynamic behavior.

The specific activity of the AEBS recovered from gel filtration was about 20–40 pmol/mg protein, and on average about 4–8-fold purification was obtained compared to the crude solubilized material. Other methods of purification, including ion exchange and affinity chromatography, have met with little success. We have found that affinity columns consisting of hydroxytamoxifen linked to agarose adsorb many proteins from gel-filtered AEBS preparations. This agrees with the results of Van Oosbree *et al.* for fractionation of rat uterine cytosol on similar columns [26].

ENDOGENOUS COMPETITORS FOR ANTIESTROGEN BINDING SITES

The question of possible endogenous ligands for AEBS arises naturally out of consideration of the origin of high affinity binding sites for the synthetic triphenylethylenes. Clark *et al.* first demonstrated AEBS inhibitory activity in boiling ethanol extracts from rat liver [27] and we have found ethanol–ether-soluble AEBS inhibitory activity in a variety of chicken tissues and in human serum [20, 22]. Figure 2 shows a TLC profile of the AEBS inhibitory activity from human serum treated with boiling ethanol, filtered and ether extracted as described by Murphy *et al.* [22]. Clearly the crude extract is heterogeneous and this is amply confirmed in subsequent experiments. A "sterol" fraction was prepared from the crude ether extract by precipitation with hexane followed by digitonin precipitation of the supernatant and combination of the two precipitated fractions. Further TLC revealed at least two classes of AEBS inhibitory activity in the "sterol" fraction and one of these was shown by GLC to contain 7-keto-cholesterol in sufficient concentration to entirely account for the AEBS inhibitory activity of the fraction [22]. Identification of the inhibitory material in the other sterol fraction has not yet been feasible.

The soluble fraction of the initial extract remaining after hexane and digitonin precipitation appears to contain fatty acids and their esters but the AEBS inhibitory components have not been identified with certainty. Huang has recently reported that unsaturated fatty acids can compete for rat liver AEBS [28]. We have also observed this in the case of the chicken (Murphy and Lazier, unpublished results).

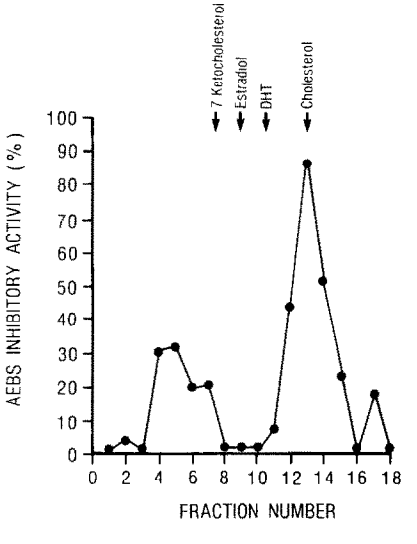

Fig. 2. AEBS inhibitory activity in the crude human serum extract. TLC was carried out in benzene–acetone (80:20) and 18 fractions were extracted with ether and tested for AEBS inhibitory activity in the chicken liver cytosol system. Reprinted from Ref. [21] with permission.

Obviously, the problem of the nature and role of putative endogenous ligands for AEBS is still a major challenge, both in terms of experimental analysis and in terms of understanding the physiological basis for the observations.

INTERACTION OF TAMOXIFEN WITH CALMODULIN

Lam[29] demonstrated that tamoxifen is a potent competitive inhibitor of Ca^{2+} calmodulin-activated phosphodiesterase activity (K_i 0.96 μM), and suggested that antagonism of calmodulin by tamoxifen might be an important mechanism in its action. If tamoxifen is inhibitory by virtue of binding calmodulin, one can speculate that calmodulin constitutes part or all of the AEBS. A fascinating connection is that the well-known phenothiazine calmodulin inhibitor trifluoperazine, binds AEBS fairly well [30]. For the chicken liver AEBS trifluoperazine and chlorpromazine cause parallel displacement of [^{3}H]tamoxifen binding with relative affinities of 4% and 0.5% respectively (Murphy and Lazier, unpublished results). If this binding is due to a calmodulin component of AEBS, one might expect it to be calcium dependent and heat denaturation resistant. However, we find that the binding is not calcium dependent and that it is distinctively temperature sensitive. Further, we have not been able to demonstrate binding of [^{3}H]tamoxifen to highly purified rat testes calmodulin (kindly supplied by Dr A. R. Means, Baylor College of Medicine). Thus it is unlikely that calmodulin is part of AEBS, but it may, of course, still play a role in the pharmacology of tamoxifen.

THE EFFECTS OF TAMOXIFEN ON SERUM LIPID PROFILES IN THE COCKEREL

A number of workers have suggested that the triphenylethylene antiestrogens may have certain estrogen-receptor-independent actions, possibly on sterol synthesis, cell growth or in cytotoxicity [31–33]. In the rat, Winneker *et al.* demonstrated an association of AEBS with plasma low-density lipoprotein particles and suggested that the sites may play some role in regulation of cholesterol metabolism [34]. As a first approach to investigating possible estrogen-independent actions of tamoxifen in birds, we have examined the effects of the drug on serum lipid profiles in cockerels [35]. Table 2 shows the influence of a single high dose of tamoxifen after 18 h on serum lipids in fed birds as measured by GLC. Pronounced decreases in free and esterified cholesterol, in phospholipid and in triglyceride levels are seen. The response is dose and time dependent and cannot be accounted for by avoidance of food by the treated animals since measurement of food intake shows no difference in the two groups (Lazier and Breckenridge, submitted for publication). The total lipid levels in very-low-density and low-density lipoproteins were reduced to 4.6 and 56% of the control values respectively. Furthermore, the decrease in phospholipid was accompanied by a distinctive change in the molecular species of phospholipids remaining, reflecting conservation of longer-chain polyunsaturated fatty acids and loss of short-chain saturated species [36].

The mechanism of these lipid-lowering effects of tamoxifen is not likely through opposition of endogenous estrogen action in the cockerels, and might instead be estrogen receptor independent. We have recently been carrying out experiments designed to address this problem using a novel diphenylmethane analogue of tamoxifen which has been shown to bind AEBS, but not the estrogen receptor, in rat liver and in MCF-7 cells [33]. This compound, [N,N-diethyl-2-[(4-phenylmethyl)]-phenoxy]-ethanamine · HCl (DPPE), was synthesized and supplied to us by Dr Lorne Brandes of the University of Manitoba[37]. Brandes and his colleagues have shown that DPPE has no estrogen agonist actively in mammalian cells but has some anti-growth effects which they suggest are mediated by AEBS acting as a histamine-type receptor [33].

In the chicken liver, DPPE binds to AEBS with affinity almost equal to that of tamoxifen, but does not bind the nuclear estrogen receptor over a wide range of concentrations (Lazier and Breckenridge, submitted for publication). Treatment of cockerels with DPPE (100 mg/kg) results in a pattern of changes in serum lipids similar to that seen after tamoxifen injection, including the drop in free and esterified cholesterol, the drop in triglycerides and in

Table 2. The influence of tamoxifen injection on serum lipid profiles*

	Vehicle-injected	Tamoxifen-treated
Free cholesterol	28.8 ± 2.4 (SEM)	14.5 ± 1.8**
Cholesterol esters	174.3 ± 8.7	123.4 ± 8.5**
Phospholipids	230.0 ± 11.3	150.6 ± 8.0**
Triglycerides	78.6 ± 14.3	40.0 ± 16.2*
Total cholesterol	134.1 ± 6.5	88.6 ± 3.7**

**$P < 0.001$. *$P < 0.05$. $n = 5$.

*Cockerels (500–600 g) were injected intramuscularly with tamoxifen citrate (100 mg/kg) in propylene glycol and after 18 h the animals were killed and serum prepared from trunk blood. Serum lipid profiles were determined by GLC. Adapted from Ref. [35].

phospholipids and the alteration in the molecular species of phospholipids. Thus it is plausible that tamoxifen and DPPE are exerting their effects through similar mechanisms, possibly involving AEBS. Even though much more work is necessary to pinpoint the exact site of the lipid-lowering actions of these drugs, such work could provide useful insights into the mechanisms of integration and regulation of lipid and lipoprotein metabolism in the chicken. At this stage, the studies permit us to propose at least that the estrogen receptor is not involved in this facet of tamoxifen action.

CONCLUSIONS

The study of tamoxifen action in the chicken has developed into a multi-faceted program involving diverse areas of biochemistry and pharmacology. The major unsolved problems are the molecular basis for the antagonistic nature of the antiestrogen–estrogen receptor complex, the identity and role of the AEBS and the spectrum of non-estrogen receptor linked actions and the mechanisms involved.

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