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Inactivation of the uterine estrogen receptor binding of estradiol during P-450 catalyzed metabolism of chlorotrianisene (TACE)

Speculation that TACE antiestrogenic activity involves covalent binding to the estrogen receptor

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Chlorotrianisene (TACE) exhibits in vitro little or no binding to the uterine estrogen receptor (ER) but demonstrates potent estrogenic activity in vivo, indicating that TACE is a proestrogen/proantiestrogen. Our earlier studies demonstrated that the incubation of TACE with rat liver microsomes and NADPH generates a reactive intermediate (T*) which binds covalently to proteins. The current study examined the possibility that T* may inactivate the uterine ER. The incubation of TACE with rat liver microsomes and NADPH in the presence of rat uteri, under conditions which generate T*, markedly decreased the binding capacity of the ER for [^{3}H]estradiol (E_2). The evidence indicates that ER inactivation was probably due to irreversible (covalent) binding of T* to the E_2 binding site. The possibility that the antiestrogenic action of TACE and of other triphenylethylenes involves such a novel mechanism is discussed.

Estrogen receptor; Metabolism; Chlorotrianisene; Tamoxifen; Methoxychlor; (Rat uterus, P-450; Rat liver)

1. INTRODUCTION

Since the discovery of the first nonsteroidal antiestrogen [1], numerous studies have been conducted to determine the mechanism of action of this class of compounds. However, despite extensive efforts in many laboratories, there is currently no mechanism which adequately explains both the agonistic and antagonistic actions of the triphenylethylene compounds [2]. Moreover, the pronounced differences in activity elicited by tamoxifen in a variety of animal species are puzzling; tamoxifen is a pure estrogen agonist in the mouse uterus, a partial agonist/antagonist in the rat uterus and a pure antagonist in the chick oviduct [3–6]. Recently it was observed that chlorotrianisene (TACE), a long-acting triphenylethylene estrogen, exhibits antiestrogenic activity [7]. These findings indicate that triphenylethylene derivatives generally share similarity of action and that the differences, such as between tamoxifen and TACE, are merely quantitative. In view of such findings, it is difficult to envision a unified mechanism of action for triphenylethylenes across animal species, based solely on the reversible binding to the estrogen receptor (ER) or to the antiestrogen binding site [17,26]. Therefore we embarked on a study of alternative mechanisms for antiestrogen action.

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Our earlier studies demonstrated that the incubation of TACE with rat liver microsomes and NADPH or with a reconstituted P-450 monooxygenase produces a reactive intermediate (T*), which binds covalently to proteins [12,13]. T* exhibits considerable stability, suggesting that T* might survive in transport. Hence, it appeared conceivable that in the course of incubation of TACE with liver microsomes and NADPH and uteri, T* would react with uterine proteins, possibly with the ER. The current study supports this supposition. The incubation of TACE with rat liver microsomes in the presence of NADPH and immature rat uteri, under conditions which generate T*, causes a pronounced decrease in the estrogen-binding sites in the uterine ER (assayed by exchange with [^{3}H]estradiol ([^{3}H]E_2)). Our studies suggest that T* reacts readily with compounds containing free sulfhydryl groups [12]. Hence, we speculate that the decrease in E_2 binding to the ER is due to the reaction of T* with cysteines at the estrogen binding site of ER [14,18]. The possibility that other antiestrogenic triphenylethylenes function by a similar mechanism, is discussed.

2. MATERIALS AND METHODS

Male and female Sprague–Dawley CD rats were from Charles River Breeding Labs (Wilmington, MA). The males (90–100 g) were treated with phenobarbital (37.5 mg/kg i.p. in 0.2 ml H_2O twice daily) for 4 days. Liver microsomes were prepared 12–15 h after the last injection [15]. Female rats (20 or 21 days old) were used for induction of uterine ornithine decarboxylase (ODC). The rats wre ovariec-

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tomized and used 5–7 days later [16]. Briefly, rats were injected with the compounds and 6 h later the uteri were removed; individual uteri were homogenized and 20 000 *g* supernate (half uterus equivalent) was incubated with [1-^{14}C]ornithine for 1 h. The reaction was terminated with 1 M citrate and the $^{14}CO_2$ was trapped on filter paper strip containing 50 μl of NCS and quantitated by scintillation spectrometry.

2.1. *Method to determine whether a compound is a proestrogen/proantiestrogen*

The procedure, previously described by us [10,11], employs in the same vessel a metabolizing system (rat liver microsomes and NADPH) and an estrogen detecting system (rat uteri). A compound is considered to be a *pro*estrogen/*pro*antiestrogen if it requires metabolism (NADPH and active microsomes) to elevate the uterine estrogen receptor (ER) in the nuclei (ER_n) and decrease the cytosolic ER (ER_c). By contrast, a compound is considered to be active per se if such distribution of uterine ER occurs without metabolism (absence of NADPH or presence of heat-inactivated microsomes). Each incubation contained 3 ml Krebs–Ringer solution (KRS), about 0.5 mg liver microsomal protein and halves of 6 uteri (sliced through the apex) and the test compound added in 5 μl ethanol; the control, lacking NADPH or containing heat-inactivated liver microsomes and NADPH, had the parallel halves of the same uteri. Prior to incubation, oxygen is bubbled vigorously through the septum into the entire mixture for 2–3 min, to preserve receptor stability [29]. The reaction is started by injecting 0.3 mg NADPH in 0.3 ml KRS through the septem (controls receive 0.3 ml KRS). Incubation is conducted for 1 h at 37°C with shaking. The uteri are washed, homogenized and the nuclei and 20000 *g* supernate are prepared. Quantitation of the estrogen receptor in the nuclei (ER_n) and in the cytosol (ER_c) was achieved by an exchange with [^{3}H]E_2 [10,11,27].

3. RESULTS AND DISCUSSION

It has been suggested that the triphenylethylenes tamoxifen and TACE are *pro*drugs [2,8,9]. We substantiated this for TACE. In vivo, TACE elicits estrogenic activity, elevating uterine weight and ODC (table 1). Similar estrogenic activity was observed with the antiestrogen–tamoxifen and with the estrogen–DES. However, in vitro, TACE did not inhibit the binding of [^{3}H]E_2 to the ER (not shown). Others also observed an extremely low binding affinity of TACE for ER_c [28]. This indicates that TACE is a proestrogen. Nevertheless, TACE exhibits some 'estrogenic' effect on the ER in vitro, causing a moderate increase in the ER_n (table 2); a similar effect was observed with tamoxifen. These effects were relatively small as compared with E_2 and DES [10]. It is unlikely that TACE and tamoxifen are metabolized by uterine monoxygenases into estrogens. Rat uteri do not exhibit significant O-demethylating activity [10,11] and, by contrast to mouse uteri [19], do not catalyze a certain aromatic hydroxylation [20]. Consequently, TACE and tamoxifen probably have weak estrogenic effects on the ER.

TACE undergoes demethylation by rat liver microsomal monooxygenase to phenolic products [21]. Hence, we anticipated that the incubation of TACE with liver microsomes will produce demethylated estrogenic metabolites which would markedly increase uterine ERn. However, it also seemed conceivable that the reactive intermediate of TACE (T*) [12,13] will bind covalently to the uterine ER, possibly at the estrogen binding site. In fact, when TACE was incubated with liver microsomes and NADPH in the presence of uteri (table 3), there was *no* increase in ER_n but instead there was a pronounced decrease in *total* uterine ER, (ER_t (ER_c plus ER_n)). There was no decrease in ER_t when NADPH was deleted or when heat-inactivated liver microsomes were used. Furthermore, there was a more pronounced decrease in ER_t with increased concentration of TACE (Fig.1). These findings indicated that TACE metabolism was required for eliciting the diminished uterine ER_t. Further studies

Table 1

Effect of treatment of ovariectomized rats with TACE, tamoxifen or DES on uterine weight and ODC

Treatment	ODC activity CO_2 evolved (pmol/uterus/h)	Uterine/body weight ×100
–	17 ± 3 (17)	38.0 ± 1.5 (17)
TACE	2089 ± 156 (6)[a]	64.2 ± 4.2 (6)[b]
Tamoxifen	1923 ± 224 (6)[a]	68.6 ± 3.5 (6)[b]
DES	2549 ± 307 (6)[a]	90.0 ± 8.5 (6)[b]

Intraperitoneal injections: TACE, 8 mg/kg BW; tamoxifen, 10 mg/kg; DES, 0.03 mg/kg. Uteri were removed after 6 h, weighed and ODC determined for each uterus. [a] $P<0.001$; [b] $P<0.05$. In parentheses, numbers of animals.

Table 2

Effect of incubations of TACE and tamoxifen with rat uteri (without liver microsomes) on the distribution of the uterine estrogen receptor in the cytosol (ER_c) and nuclei (ER_n)

Compound	[^{3}H]E_2 bound/uterus (fmol) ER_c	ER_n	ER_c + ER_n
TACE, 2 μM[a]	663 ± 124	268 ± 29*	931
Tamoxifen, 2 μM[b]	472	271	743
Control[c]	887 ± 45	82 ± 8	969 ± 54

[a] Values represent mean ± SE (3 experiments). [b] Values represent a single experiment. [c] Values represent mean ± SE (7 experiments). * $P<0.001$.

Table 3

Effect of incubation of TACE with rat liver microsomes and uteri on the uterine nuclear (ER_n) and cytosolic (ER_c) estrogen receptor

TACE (μM)	NADPH	[^{3}H]E_2 bound/uterus[a] (fmol) ER_c	ER_n	ER_c+ER_n
0.2	+	204.3±45.5 (6)*	138.2± 6.7 (8)	378.7±28.7 (6)*
0.2	–	610.8±42.8 (6)	95.9±11.2 (8)	715.4±47.9 (9)
0.2	+ (boiled microsomes)	680.8 (2)	121.7 (2)	802.4 (2)

[a] Values represents mean ± SE. Number of experiments in parentheses.
* $P<0.001$.

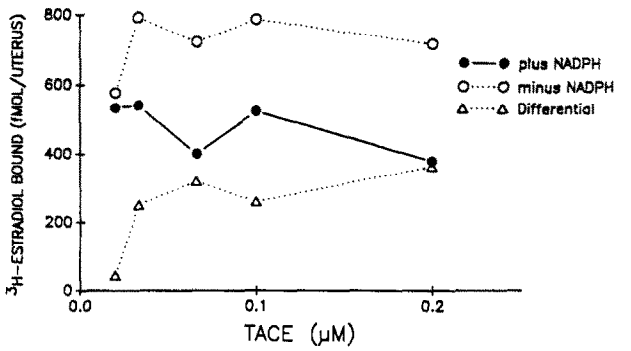

Fig.1. The effect of incubation of various concentrations of TACE with rat liver microsomes and uteri on total uterine ER (ER_t).

on whether the diminished ER_t reflects a preferential decrease in ER_c or ER_n might yield information or ER trafficking.

The pesticide methoxychlor is metabolized by liver microsomes into demethylated estrogenic products [10,11,31]. We previously observed that methoxychlor, like TACE, can be metabolically activated to bind covalently to proteins [22,23]. However, by contrast to TACE [13], the reactive intermediate of methoxychlor (M*) did not bind to the exogenously added albumin (unpublished result). Therefore, M* was not expected to react with distant sites, such as uterine ER. In fact, the microsomal metabolism of methoxychlor increased ER_n and decreased ER_c without diminishing ER_t [10,11] (table 4). Similarly MDDE, and in vivo metabolite of methoxychlor, did not affect ER_t. Thus we concluded that whereas T* could inactivate ER, M* could not. Also this demonstrated that ER inactivation was not due to formation of other factors, unrelated to TACE metabolism.

The decrease of ER_t during the generation of T* was shown by the diminished binding of [^{3}H] E_2 in the exchange assay. The possibility that TACE is metabolized

Table 4

Effect of hepatic monooxygenase mediated metabolism of TACE, methoxychlor and MDDE on the subcellular distribution of the uterine estrogen receptor in cytosol (ER_c) and nuclei (ER_n)

Compound	Microsomal system	[^{3}H]E_2 bound/uterus (fmol) ER_c	ER_n	$ER_c + ER_n$
TACE[a]	Active	248 ± 91*	133 ± 17	381 ± 96*
	Inactive	610 ± 134	211 ± 8	821 ± 134
Methoxychlor[b]	Active	986	262	1248
	Inactive	1211	89	1300
MDDE[b]	Active	359	375	734
	Inactive	696	97	793

Each compound was at 2 μM. Active system, composed of PB rat liver microsomes (0.5 – 0.7 mg prot.). Inactive system lacked NADPH (TACE adn MDDE) or contained NADPH and heat-inactivated microsomes (methoxychlor). MDDE, 1,1-dichloro-2,2-bis(*p*-methoxyphenyl)ethene. [a] Values represent mean ± SE (3 experiments). [b] Represent mean of two experiments.

* $P<0.05$.

to a product with much higher affinity for the ER than E_2 and thus interferes with the exchange of [^{3}H]E_2 is unlikely: (i) where unable to isolate such a metabolite from incubations of TACE with liver microsomes and (ii) increasing the concentration of [^{3}H]E_2 5-fold and stripping the uterine cytosol with charcoal prior to the exchange assay did not alter the results (not shown). Currently, we have no information on whether the steroid-binding site is masked by T* or merely has become altered. Our previous studies suggest that T* binds to the cysteine sulfhydryl in proteins ([12] and unpublished data). Cysteine is present at the steroid-and DNA-binding sites of the ER [14,18]. Therefore it is possible that T* binds to the cysteines of the ER and interferes with E_2 binding. An alternate, albeit less likely, interpretation of these results is that the metabolism of TACE has diminished the receptor level, by increasing its processing or by altering its structure possibly through a non-covalent binding mechanism. Nakao et al. [24] observed that the administration of tamoxifen to rats causes a dramatic decrease in uterine cytosolic estrogen-binding sites and elicits a modified 'receptor'. This tempts a speculation that tamoxifen binds covalently to the receptor in vivo. Our preliminary findings that tamoxifen undergoes metabolic activation by rat liver microsomes, resulting in covalent binding to protein (not shown), supports such a possibility. Previously, we demonstrated that the pretreatment of ovariectomized rats with tamoxifen abolishes the induction of ODC by a subsequent treatment with E_2 [25]. Thus, it is conceivable that this desensitization might occur by a tamoxifen mediated inactivation of the ER. Whether TACE also could cause a desensitization of ODC induction, is not known.

In conclusion, the current studies suggest a novel mechanism to explain the agonistic and antagonistic activity of triphenylethylenes. We propose that the two effects are the resultant of the reversible binding of estrogenic metabolites and of the reactive intermediate (RI). The dramatic species differences in response to tamoxifen could be due to qualitative or quantitative differences in RI formation, possibly because of species differences in P-450 isoforms, or due to species differences in quenching the RI. It would be of particular interest to determine whether the irreversible binding causes a partial or complete inactivation of the receptor. Alternatively, it is possible that the covalent binding of TACE merely yields a persistently activated, albeit altered receptor, a mechanism proposed for the reaction of 16α-hydroxyestrone [30]. The examination of these possibilities would require extensive investigations.

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