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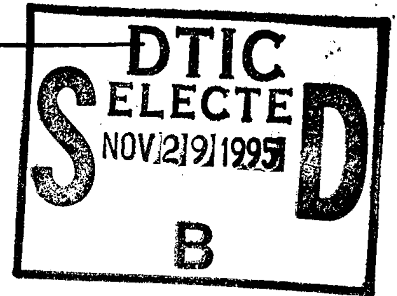

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13. ABSTRACT *(Maximum 200 words)*

The Ah receptor (AhR), a gene regulatory protein, has been implicated to play a significant role in the development and/or progression of mammary tumors in humans and animals. We further hypothesize that it plays a role in the normal development of breast tissue. We will define the cell- and developmentally-related expression and activation of the AhR in mammary tissue, determine the ability of AhR agonists to affect the expression of the estrogen receptor (ER) in this tissue, and define the mechanism(s) by which these events occur. We have shown that the human ER structural gene contains specific DNA sequences that binds activated human AhR under conditions *in vitro*. We have sought to establish an *in vivo* model to further investigate the mechanisms whereby the AhR may regulate the ER gene. In contrast to previous investigations by others, we found that the AhR agonist, TCDD, did not affect either ER levels or estrogen-induced responses in the weanling female rat. These data indicate that age and/or developmental period play a crucial role in the ability of TCDD, *via* the AhR, to modulated the ER and estrogen-mediated responses. We are progressing on proposed studies to examine the developmental expression of the AhR in rat mammary tissue and the effects of AhR agonists on ER expression. We have also determined that a gene encoding an enzyme intimately involved in steroid hormone metabolism, 3β-hydroxysteroid dehydrogenase isomerase, is affected by AhR agonists. We will further examine the regulation of this gene in breast tissue.

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A. INTRODUCTION

The estrogen receptor (ER) and the response of this receptor to estrogenic compounds influence not only the molecular events that account for the development and progression of breast cancer, but also the response of this disease to a variety of therapeutic measures. As such, it is important to determine what factors may regulate the expression and actions of this receptor in breast tissue. Previous results have shown that the activation of another transcription factor, the Ah receptor (AhR), influences the spontaneous generation of mammary tumors in laboratory animals, alters estrogen-induced responses, and affects the ER content of tissues. Certain xenobiotics, e.g. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and structurally-related compounds, have been shown to activate the AhR to a DNA-binding state and elicit a variety of AhR-mediated biochemical and biological responses. Although the exact normal function of the AhR and its endogenous ligand are not known, the biological responses which TCDD and the halogenated aromatic hydrocarbons elicit indicate this protein serves some normal role in the control of processes involved in tissue differentiation and proliferation.

We hypothesize that the AhR plays an important role in the normal development of breast tissue. Furthermore, the abnormal regulation of the activity of this transcription factor may play a critical role in breast tumor development. We believe that AhR agonists, such as TCDD, cause down-regulation of the ER *in vivo* by binding to regulatory regions of the ER structural gene and decreasing the rate of its transcription.

The objectives of this research are three-fold: **1)** to determine the cell- and developmentally-related expression and activation of the AhR in rat mammary tissue, **2)** to examine the ability of AhR agonists to affect the expression of the ER in mammary tissue; and **3)** to examine the mechanism by which the AhR down-regulates the expression of the ER structural gene.

B. BODY

B.1. Examine the mechanism by which the AhR down-regulates expression of the ER structural gene. We have shown previously that the human ER structural gene contains specific DNA sequences (DREs, dioxin-responsive elements) that bind activated mouse and human AhR under conditions *in vitro* (White & Gasiewicz, 1993). We proposed that the AhR may regulate ER expression through the direct modulation of gene transcription. To prove this, it is necessary to show that this

regulation occurs under whole cell and/or *in vivo* conditions. Therefore, the initial goal was to establish a model to further investigate the mechanism of this down-regulation.

In our initial experiments in collaboration with Dr. A. Notides and using antibodies directed against the ER, we found that TCDD did not cause a decreased expression of the ER protein in human breast MCF7 cells. However, others have reported an effect on ER protein *in vivo* (Astroff and Safe, 1988; Romkes and Safe, 1988; Astroff et al., 1990; Hruska and Olson, 1989). Furthermore, it is likely that the particular response may be a function of tissue-, cell-, and/or temporal-specific ratios of various transcription factors. Therefore, we sought to establish a rat model to further investigate the mechanism for the antiestrogenic effects of TCDD.

TABLE 1

Effect of Estradiol and TCDD on Uterine, Thymic, and Hepatic Weights in Weanling Female Rats [a]

Treatment groups	Uterus weight (mg/kg BW)[b]	Thymus weight (mg/kg BW)	Liver weight (% BW)
Control (n=19)	0.73 ± 0.19	3.56 ± 0.29	4.58 ± 0.34
Estradiol (E_2) (n=20)	1.60 ± 0.23 [c]	3.59 ± 0.79	4.61 ± 0.16
E_2 + 20 µg/kg TCDD (n= 12)	1.68 ± 0.22 [c]	2.42 ± 0.29 [d]	5.44 ± 0.55 [d]
E_2 + 40 µg/kg TCDD (n=19)	1.61 ± 0.24 [c]	1.95 ± 0.26 [d]	5.49 ± 0.58 [d]
E_2 + 80 µg/kg TCDD (n=20)	1.74 ± 0.27 [c]	2.21 ± 0.50 [d]	5.49 ± 0.58 [d]

[a] Rats were treated sc with olive oil or TCDD on day 19, followed by treatment on days 21 and 22 with olive oil or 10 µg/kg/day E_2, as shown. All rats were euthanized on day 23.

[b] Results are the mean ± SD of a total of n animals from three separate experiments.

[c] Significantly different from control, $p < 0.001$.

[d] Significantly different from corresponding controls and E_2 treated, $p \leq 0.001$.

The effects of estradiol (E2) alone or TCDD plus E2 on several E2-dependent parameters were evaluated in weanling female Sprague-Dawley rats. E2 treatment (10 µg/kg/day at post-natal days (PND) 22 and 23) caused significant increases in relative uterine weight (Table 1) and keratinization of the vaginal epithelium (Fig. 1). E2 treatment significantly reduced uterine ER protein levels and serum FSH levels (Table 2), with a trend toward reduction of ER mRNA levels (Fig. 2). None of these parameters were affected by pretreatment with TCDD at PND 19. Uterine

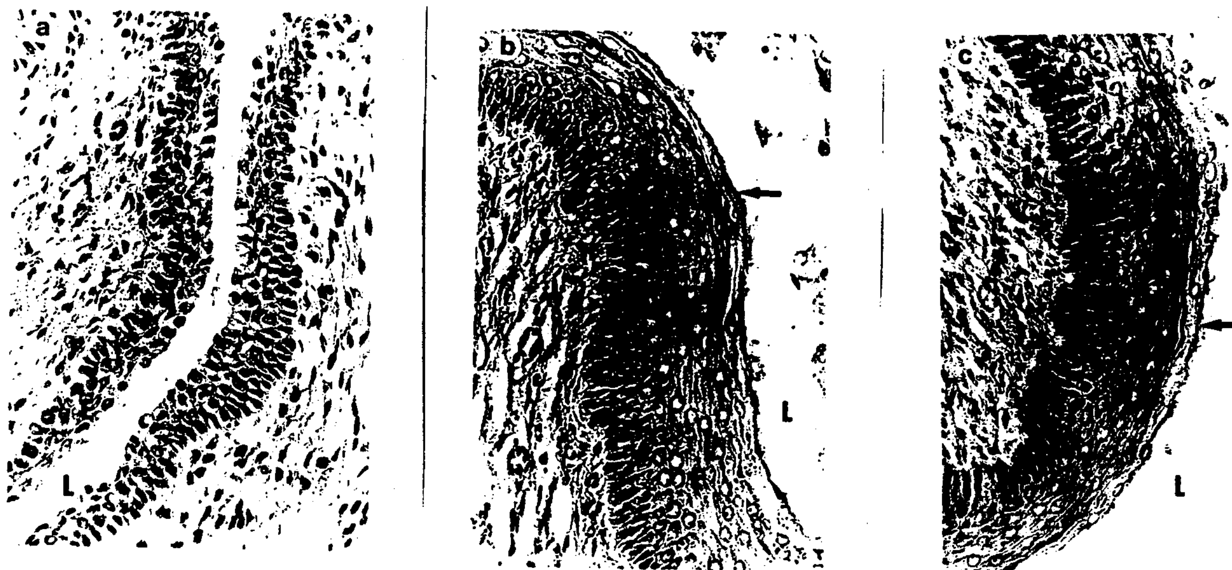

Fig. 1. Photomicrographs of vaginal epithelia. Rats were injected sc with olive oil or 10 μg/kg/day E2 on days 21 and 22, or E_2 (days 21 and 22) plus a pretreatment of TCDD (20, 40, or 80 μg/kg) on day 19. All animals were euthanized on day 23. Vaginal epithelium (E) from **(a)** a control rat, showing a transitional morphology; **(b)** an E_2-treated rat, showing a stratified, squamous morphology and producing abundant amounts of keratin (arrow); or **(c)** a rat treated with E_2 plus 80 μg/kg TCDD, also showing a keratinizing epithelium. Results were confirmed in two separate experiments, or a total of 8 animals per group (4 animals in the 20 μg/kg TCDD dose group). L, vaginal lumen; 310 x magnification.

TABLE 2
Effects of E2 and TCDD on Uterine Estrogen Receptor and Progesterone Receptor Content and Serum FSH Levels [a]

Treatments	Estrogen receptor (fmol/mg protein)[b]	Progesterone receptor (fmol/mg protein)[c]	Serum FSH (ng/ml)[d]
Control	632.8 ± 164.7 (7) [e]	191.2 ± 24.8 (7)	6.8 ± 2.4 (17)
E_2	404.9 ± 63.8 (8) [f]	188.3 ± 86.7 (8)	4.1 ± 1.1 (19) [g]
E_2 + 20 TCDD	472.9 ± 117.3 (4)	183.6 ± 29.5 (4)	3.7 ± 1.0 (10) [g]
E_2 + 40 TCDD	418.3 ± 122.2 (7) [h]	193.9 ± 94.0 (7)	5.1 ± 1.8 (18) [i]
E_2 + 80 TCDD	459.9 ± 99.1 (8) [h]	208.3 ± 83.7 (8)	4.3 ± 1.3 (15) [g]

[a] Rats were treated as in Table 1. Dose levels of TCDD are in μg/kg.
[b] Estrogen receptor levels were analyzed by EIA.
[c] Progesterone receptor levels were analyzed using a ligand binding assay.
[d] FSH serum levels were analyzed by RIA.
[e] All values are expressed as means ± SD for (n) number of animals.
[f] Significantly different from control, $p = 0.003$.
[g] Significantly different from control, $p \leq 0.002$.
[h] Significantly different from control, $p < 0.03$.
[i] Significantly different from control, $p = 0.03$; significantly different from E_2 treated, $p = 0.043$.

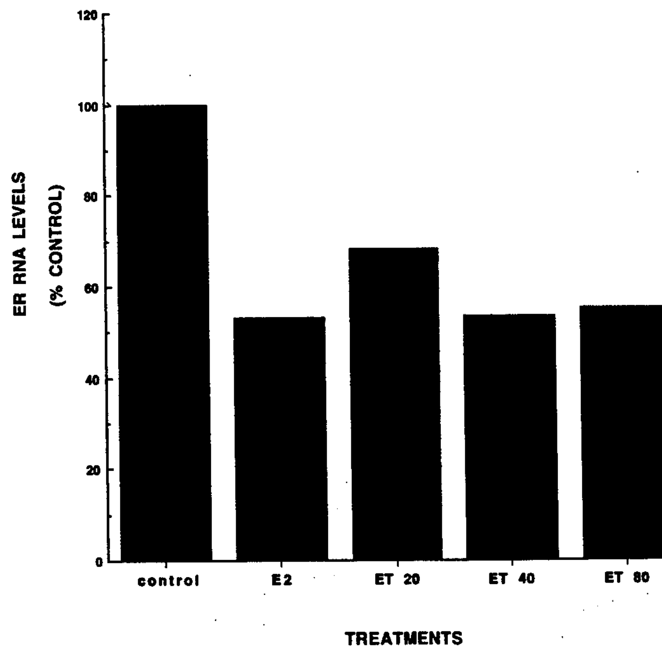

Fig. 2. Estrogen receptor mRNA levels. Animals were treated as in Fig. 1. Pairs of uteri within a treatment group were pooled, and total RNA was isolated, serially diluted onto a Nytran membrane, and analyzed using a slot blot hybridization procedure. For each RNA sample, values were initially plotted as PhosphorImager units vs amount of RNA loaded. Mean PhosphorImager values for 10 µg total RNA were calculated from the linear portions of the curves. Values are expressed as percentages of control and represent the mean from two RNA samples per treatment from one experiment. Treatments: E_2, estradiol alone; ET 20, E_2 + 20 µg/kg TCDD; ET 40, E_2 + 40 µg/kg TCDD; ET 80, E_2 + 80 µg/kg TCDD.

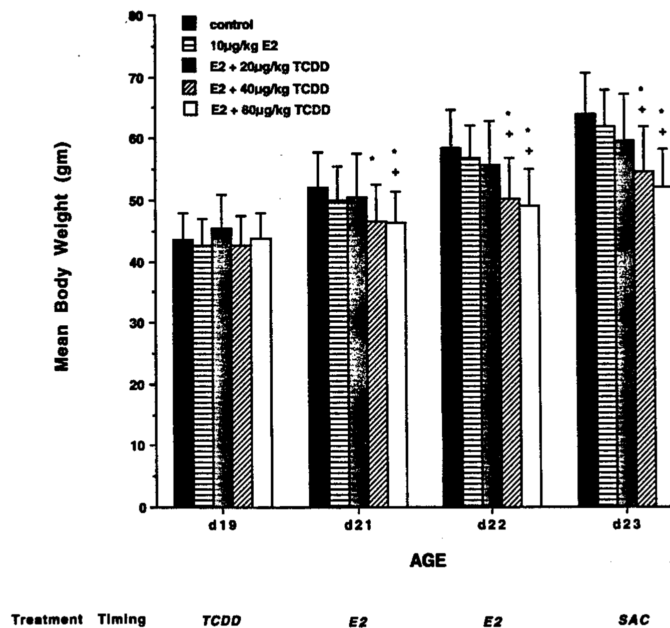

Fig. 3. Body weights of animals throughout the experiment. Animals were treated as in Fig. 1 and weighed on the day of each injection and at the time of euthanasia. Days of injection with either E_2 or TCDD are indicated. * Significant difference from control, $p < 0.01$; + significant difference from E_2 treatment, $p \leq 0.002$.

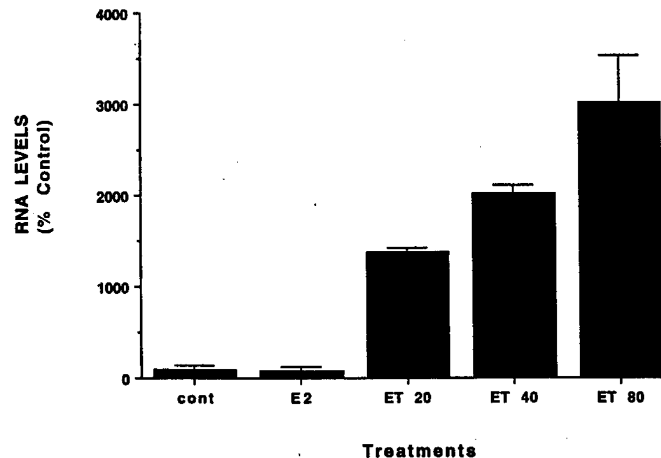

Fig. 4. CYP1A1 mRNA levels. Animals were treated as in Fig. 1. Total hepatic RNA was isolated from single liver samples, and slot blots were prepared. Mean PhosphorImager units were calculated as in Fig. 2. Values are expressed as percentages of control and represent the results of one experiment for a total of two samples per treatment group.

progesterone receptor levels were not affected by E2 or TCDD in this study (Table 2). In contrast, TCDD significantly decreased body weight by PND 21 (Fig. 3), significantly decreased relative thymic weights (Table 1), and signficantly increased hepatic weights (Table 1). In addition, TCDD dramatically induced hepatic cytochrome P450 1A1 mRNA levels (Fig. 4), indicating that TCDD was properly delivered and could mediate other well-documented AhR-dependent events. (The details of these results are contained in a recent publication (White et al., 1995) included in the Appendix.

In light of previous investigations where an effect of TCDD on E2-induced uterine weights and ER proteins levels were observed, we interpret our data to indicate that age and/or developmental period plays a crucial role in the ability of E2 to elicity biological effects, as well as the ability of TCDD via the AhR to interfere with these effects. Thus, while we, at least in this study, were unsuccessful in establishing a model to examine the effects of TCDD in the rat, the results are significant in pointing out the likely developmental specificity for the actions of the AhR.

While we were in the process of performing these studies, a group of other investigators established that treatment of a human ovarian carcinoma cell line, BG-1, with TCDD results in a dose-related depression of ER mRNA and protein (Clark, 1995). Some of our future experiments will focus on this cell line to examine mechanisms of TCDD-elicited down-regulation of the ER.

B.2. Determine the cell- and developmentally-related expression and activation of the AhR in rat mammary tissue. Despite data indicating an affect of AhR agonists on the spontaneous generation of mammary tumors, the expression and cellular distribution of the AhR in this tissue has not been examined. These studies will provide important information suggesting a period in which the AhR may play a critical role in breast tissue development.

To date we have been working on the methodologies for immunohistochemistry of the AhR protein and *in situ* hybridization for the determination of AhR mRNA. Some difficulty has been enountered with the availability of appropriate anti-AhR antibodies. We will be continuing the development of these procedures within the coming funding period, and will examine the developmental expression of the AhR in rat mammary tissue. We are however, continuing studies to quantitate the presence of AhR in mammary tissue and changes with age, pregnancy and lactation. These results will be reported at a later period.

B.3. Examine the ability of AhR agonists to affect the expression of the ER in mammary tissue. These studies will complement the previous objective by suggesting a relationship between AhR expression in this tissue and the regulation of ER protein and mRNA expression as affected by AhR agonists. These studies are very dependent on those in the previous objective. Furthermore, the studies presently underway as outlined in B.1. will also assist in designing the protocols for these. As such, we have not yet begun these studies, but will start these in the upcoming year.

However, other work in our laboratory has established that TCDD via the AhR alters the expression of a particular gene important in steroid hormone metabolish, 3β-hydroxysteroid dehydrogenase isomerase (Kent and Gasiewicz, submitted). At present we do not know if this may be altered in breast tissue or if this alteration may significantly alter steroid, particularly E2, action in this tissue. While we are performing the above studies to examine for the effect of TCDD on ER expression in breast tissue, we will also examine for any effect on 3β-hydroxysteroid dehydrogenase isomerase expression.

C. CONCLUSIONS

The results to date are important in emphasizing the tissue- and age-dependent nature of the effects of TCDD on E2-dependent responses. Thus, these data indicate that if the AhR does have some role in regulating the ER and ER-dependent responses, it is likely to be developmental specific. This in fact could have important implications for the normal and abnormal development of breast tissue.

Future work will specifically focus on breast tissue for the presence and actions of the AhR specifically as it may regulate the expression and actions of the ER (and other genes affecting the actions of E2 in this tissue).

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E. Appendix.

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