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Evidence That Atrazine and Diaminochlorotriazine Inhibit the Estrogen/Progesterone Induced Surge of Luteinizing Hormone in Female Sprague-Dawley Rats Without Changing Estrogen Receptor Action

Tami S. McMullin,* Melvin E. Andersen,*[1] Alan Nagahara,† Trent D. Lund,† Toni Pak,† Robert J. Handa,† and William H. Hanneman*[,2]

Department of Environmental and Radiological Health Sciences, and †Department of Biomedical Sciences, Colorado State University, Fort Collins, Colorado 80523

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High oral doses of atrazine (ATRA) disrupt normal neuroendocrine function, resulting in suppression of the luteinizing hormone (LH) surge in adult, ovariectomized (OVX) estrogen-primed female rats. While the mechanism by which ATRA inhibits LH secretion is not known, current data indicate that ATRA does have anti-estrogenic properties *in vitro* and *in vivo*. In the body, ATRA is rapidly converted to diaminochlorotriazine (DACT). The present study was conducted to investigate the effects of ATRA and DACT on the estradiol benzoate (EB)/progesterone (P) induced LH surge and to determine if such changes correlate with impaired estrogen receptor (ER) function. ATRA, administered by gavage for five consecutive days to adult OVX, female Sprague-Dawley rats, caused a dose-dependent suppression of the EB/P induced LH surge. Although to a lesser degree than ATRA, DACT significantly suppressed total plasma LH and peak LH surge levels in EB/P primed animals by 60 and 58%, respectively. DACT treatment also decreased release of LH from the pituitary in response to exogenous gonadotropin releasing hormone (GnRH) by 47% compared to control. Total plasma LH secretion was reduced by 37% compared to control, suggesting that in addition to potential hypothalamic dysfunction, pituitary function is altered. To further investigate the mechanism by which hypothalamic function might be altered, potential anti-estrogenicity of ATRA and DACT were assessed by evaluating ER function treated rats. Using an *in vitro* receptor binding assay, ATRA, but not DACT, inhibited binding of [^{3}H]-estradiol to ER. In contrast, ATRA, administered to female rats under dosing conditions which suppressed the LH surge, neither changed the levels of unoccupied ER nor altered the estrogen induced up-regulation of progesterone receptor mRNA. Collectively, these results indicate that although ATRA is capable of binding ER *in vitro*, the suppression of LH after treatment with high doses of ATRA is not due to alterations of hypothalamic ER function.

Key Words: **atrazine; chlorotriazines; estrogen receptor; DACT; brain; hypothalamus.**

Chlorotriazine (Cl-TRI) compounds such as atrazine (ATRA) are widely used herbicides throughout the United States. ATRA and other Cl-TRIs have been reported to disrupt the estrous cycle in various laboratory rat strains, including Sprague-Dawley (SD) rats (Cooper *et al.*, 1996). In other animal studies, lifetime exposure to high levels of ATRA and other Cl-TRIs cause premature reproductive senescence in addition to early onset and increased incidence of mammary tumors in female SD rats (Stevens *et al.*, 1994).

In addition to the carcinogenic effects, Cl-TRIs elicit a broad range of effects associated with neuroendocrine disruption. These include disruption of normal ovarian cycling patterns in adult female, ovariectomized (OVX) rats (Peruzovic *et al.*, 1995), delayed puberty in male rats (Stoker *et al.*, 2000), and prenatal developmental effects such as suppression of suckling induced prolactin (PRL) release (Stoker *et al.*, 1999). In addition to its effects on PRL release, ATRA is capable of suppressing the LH surge in adult, OVX, estrogen primed female rats (Cooper *et al.*, 2000) at doses of ATRA ranging from 50–300 mg/kg administered from 1 to 21 days.

The mechanism by which ATRA suppresses the LH surge is not known. While multiple neuroendocrine signals and neurotransmitters affect LH release from the pituitary, it is largely regulated by estrogen binding to estrogen receptor (ER) in various hypothalamic nuclei (Herbison, 1998; Kalra, 1993; Legan *et al.*, 1975). Increasing circulating estrogen levels on the morning of proestrus initiate a positive feedback response which drives gonadotropin releasing hormone (GnRH) release and results in an LH surge. *In vitro* and *in vivo* studies suggest that ATRA is not estrogenic (Eldridge *et al.*, 1994). However, ATRA exhibits anti-estrogenic properties in rat uterus (Tennant *et al.*, 1994a), in estrogen responsive MCF-7 cell lines (Tran *et al.*, 1996), and decreases estrogen binding to ER in uterine cytosol (Tennant *et al.*, 1994b). The importance, if any,

[1] Present address: CIIT – Centers for Health Research, Six Davis Drive, PO Box 12137, Research Triangle Park, NC 27709.

[2] To whom correspondence should be addressed at Department of Environmental and Radiological Health Sciences, Physiology Building (1680) Room 132, Fort Collins, CO 80523-1680. Fax: (970) 491-7569. E-mail: hanneman@colostate.edu.

of anti-estrogenic actions of the triazines for toxic responses in the intact animal remains a topic of current debate.

Once in the body, ATRA is extensively metabolized to the mono- and di-dealkylated chlorinated metabolites and to non-chlorinated, glutathione conjugate metabolites. Although limited, studies show that Cl-TRIs can elicit common neuroendocrine effects. This is exemplified by studies in male Wistar rats that show alterations in puberty and thyroid function (Stoker *et al.*, 2002). As a result of these and other data, U.S. EPA risk assessment guidelines for triazines consider ATRA and its Cl-TRI metabolites to be toxicologically equivalent in terms of their effects on the neuroendocrine system.

Our group has recently shown that DACT is the major chlorinated metabolite in the plasma after dosing with ATRA, accounting for over 95% of the total chlorotriazine plasma area under the curve (AUC). Twenty-four hours after dosing with 300 mg ATRA/kg, the AUC for ATRA is 2.93 mg/l-h whereas the AUC for DACT is 486.09 mg/l-h (McMullin *et al.*, 2003). These data indicate that *in vivo* treatment with ATRA produces minimal tissue concentrations of ATRA and high tissue concentrations of DACT, making exposure of the brain to ATRA unlikely. Although the brain is most likely being exposed to DACT, not ATRA, there is limited information on the mechanistic behavior of DACT.

In this study the relative potency of DACT was compared to ATRA in terms of its ability to suppress the EB/P induced LH surge and in altering pituitary and/or hypothalamic function. Additionally, we examined the ability of these compounds to interfere with estrogen binding to ERalpha (ERα) *in vitro* and *in vivo*. *In vivo*, changes in estrogen mediated responses in the hypothalamus were examined. These changes included evaluation of unoccupied levels of ER and estrogen induced progesterone receptor (PR) mRNA expression in two estrogen responsive hypothalamic nuclei associated with reproductive function, the anteroventral periventricular nucleus (AVPV) and the medial preoptic nucleus (MPOA). The results of these studies indicate that ATRA and/or DACT are not likely to modulate LH through regulation of ERα.

MATERIALS AND METHODS

Animals. Female SD rats (225–275 g; Charles River Laboratories, Raleigh, NC) were shipped to Colorado State University and housed in the animal care facility with a 12 h light/12 h dark (0600/1800 h) photoperiod. Food and water were available *ad libitum.* All surgical and experimental procedures were approved by Colorado State University Animal Care and Use Committee.

Treatment. Technical grade ATRA (2-chloro, 4-ethylamino, 6-isopropylamino-s-triazine) of 97.1% purity and DACT of 96.8% purity (generously provided by Syngenta Corporation, Greensboro, NC) were utilized in these studies.

Effects of ATRA, or Its Metabolite DACT, on the LH Surge in Adult, Female OVX Rats

Estrogen priming. Rats were treated with either 300 mg ATRA/kg bw in a 0.5% carboxymethylcellulose (CMC) suspension or an equal volume of 0.5% CMC via oral gavage between 0900 to 1000 h for five consecutive days. Rats were bilaterally OVX on day 0. On day 4, an indwelling right atrial catheter was inserted via the jugular vein and exteriorized at the nape of the neck similar to previous protocols (Jongen and Norman, 1987; Levine and Ramirez, 1982). This procedure was followed by insertion of a Silastic capsule containing 17β estradiol (5 mm long, 0.078″ i.d., 0.125″ o.d.) into the subcutaneous (sc) pocket. On the fifth and sixth days of dosing, serial blood samples (0.3 ml) were collected at 1200, 1400, 1500, 1600, 1700, 1800, and 1900 h into heparinized syringes. Serial blood samples were collected remotely via the indwelling right atrial catheters connected to PE50 tubing to allow the animals to remain in their home cage. Animals were heparinized with 14 units of sodium heparin prior to sampling. The samples were centrifuged and plasma was removed and frozen at –20°C until assayed for LH. To maintain hematocrit, red blood cells (RBCs) were resuspended in saline and reinfused into each animal prior to the subsequent bleed. After completion of the experiment, rats were anesthetized and decapitated.

Estradiol benzoate/progesterone (EB/P) priming in OVX female rats. These studies determined if exposure to ATRA or DACT alters the LH surge. To mimic the endogenous hormonal milieu prior to an LH surge, adult female rats were primed with both EB and P. Bilaterally OVX and right atrial cannulated adult, female SD rats were purchased from Charles River Laboratories and acclimated for one week prior to dosing. Animals were exposed to 300, 100, 30, or 0 mg ATRA/kg or 77 mg DACT/kg in a 0.5% CMC suspension via oral gavage between 0900 to 1000 h for five consecutive days. The rats were injected sc with EB dissolved in safflower oil vehicle (10 μg/100 g bw) for three consecutive days (days 2–4) simultaneously with dosing of ATRA or DACT. On day 5, P (2 mg/animal in safflower oil vehicle) was injected sc between 1030 and 1100 h. Serial blood samples were collected remotely via the indwelling right atrial catheters connected to PE50 tubing to allow the animals to remain in their home cage. Animals were heparinized with 14 units of sodium heparin prior to sampling. Samples (0.3 ml) were collected at 1200, 1400, 1500, 1600, 1700, 1800, 1900, and 2000 h. To maintain hematocrit, RBCs were resuspended in saline and reinfused into each animal prior to the subsequent bleed. The samples were centrifuged and plasma was separated and frozen at –20°C until assayed for LH. After completion of the experiment, rats were euthanized with CO_2.

LH radioimmunoassay (RIA). Plasma LH levels were determined by RIA using methods previously described (Pak *et al.*, 2001). All samples were run in duplicate in a single assay. rLH RP-3 and rLH-S11 were obtained from the National Hormone and Peptide Program and used as the RIA standard and primary antibody, respectively. oLH was iodinated by the Colorado State University peptide analysis facility using the chloramine-T method and used as the trace hormone. The detection limit of the assay was 1.2 ng/ml. The intrassay and interassay coefficients of variation were 5.2 and 8.3%, respectively.

Effect of DACT on Pituitary LH Release after GnRH Challenge

The purpose of this experiment was to examine pituitary function, as indicated by its ability to release LH in response to GnRH, under dosing conditions of DACT which suppress the EB/P primed LH surge. Hormone priming and dosing with DACT were identical to that described for the EB/P primed animals. Serial blood samples were collected remotely via the indwelling right atrial catheters connected to PE50 tubing to allow the animals to remain in their home cage. Animals were heparinized with 14 units of sodium heparin prior to sampling. Beginning at 1200 h, serial blood samples (0.2 ml) were collected every 10 min. Two baseline samples were collected prior to an iv bolus injection of 100 ng GnRH /100 g bw (Peninsula Laboratories, Inc.; Lot # 036833) and six samples were taken every 10 min thereafter. Plasma was analyzed for LH via RIA. The samples were centrifuged and plasma was separated and frozen at –20°C until assayed for LH as described above. Upon completion of the experiment, rats were euthanized with CO_2.

The Effect of ATRA or DACT on Estradiol Binding to ER in Vitro

Previous studies indicate that ATRA has anti-estrogenic properties. To further investigate the mode of action by which ATRA and DACT might be

suppressing the LH surge, the ability of ATRA or DACT to alter binding of estradiol to estrogen receptor alpha (ERα) was evaluated.

Chemicals and reagents. Solutions of ATRA were prepared in absolute ethanol (solubility = 83.3 mM) at a concentration of 6 mM. Primary stock solutions of DACT were in DMSO at a concentration of 6 mM. The solubility of ATRA were verified by GC/MS spike recovery experiments which showed 98% recovery (data not shown). TEGM stock buffer (10 mM Tris; 1.5 mM EDTA; 10% glycerol; 25 mM molybdate; pH 7.4 on ice) was stored at 4^0C. TEGMD buffer was made the morning of the assay by adding 1.0 mM dithiothreitol to TEGM buffer. All buffer reagents were purchased from Sigma-Aldrich Co., St. Louis, MO. [^{3}H]estradiol stock (New England Nuclear; NET-317; 71 Ci/mmol) was diluted with ethanol to 25 ml and stored at -70^0C until further diluted in TEGMD to the appropriate concentration for the binding study.

Uterine cytosolic ER binding assay. Uteri from two adult, OVX female SD rats (Charles River Laboratories) were dissected and fat was removed. Uteri were minced and homogenized in 6 ml of ice cold TEGMD buffer with a glass homogenizer (Dounce Co., Vineland, NJ). Homogenates were centrifuged at 100,000 × *g* in an ultracentrifuge (Beckman Coulter, Inc., Palo Alto, CA) with a fixed angle rotor (Sorvall TI 60, Dupont-Sorvall, Wilmington, DE) for 15 min at 4°C to prepare a cytosolic fraction. To evaluate dose-dependent binding, 100 μl of uterine cytosol were incubated with increasing concentrations of ATRA (0 to 0.75 mM) in TEGMD buffer. ATRA was allowed to incubate in the cytosolic mixture for either 0, 15, 30, or 60 min prior to addition of [^{3}H]estradiol in order to examine the ability of ATRA to inhibit [^{3}H]estradiol binding to ER. After the appropriate pre-incubation period, 1 nM [^{3}H]estradiol was added to each tube. The tubes were capped, vortexed, and incubated for 2 additional hours at RT. To determine nonspecific binding, parallel incubation tubes also included a 200-fold excess of diethylstilbesterol (DES). All incubations were performed in triplicate.

Free and bound radioligand were separated using 1 ml lipophilic Sephadex LH-20 columns as previously described (Handa *et al.*, 1986). Two-hundred μl of TEGMD buffer was added to the top of each column and allowed to equilibrate and drip dry. 127.5 μl of each sample was placed on a Sephadex column, allowed to enter the column and then followed by 100 μl of TEGMD. After 30 min incubation on the column, each column was flushed with 300 μl of TEGMD twice. Scintillation vials were placed under each column and each column was allowed to drip dry overnight at 4^0C. Four ml of scintillation fluid (Packard Ultima Gold) were added to each vial. Vials were capped, shaken, and counted (5 min/vial) on a 2900 TR Packard scintillation counter (Packard Bioscience, Meriden, CT).

ERα binding assay. Full length rat ERα was translated from a DNA template (pcDNA ERα; RH Price, UCSF) using the TnT® Coupled Reticulocyte Lysate translation procedure (Promega Corporation; Madison, WI) with T7-RNA polymerase, during a 90 min reaction at 30°C. Translation reaction mixtures were stored at −80°C until further use.

Briefly, ATRA and DACT at increasing concentrations (0–1.0 mM) were incubated with 1.0 nM of [^{3}H]estradiol in 100 μl of ERα an equal protein concentrations per tube. All tubes were brought to a total volume of 150 μl with TEGMD buffer. All tubes were capped, vortexed, and incubated for 3 h at RT. Separation of free and bound were as described above.

Effects of ATRA Exposure on Estrogen-Mediated Effects in Two Estrogen Responsive Hypothalamic Nuclei

Two indicators of ER activity in the hypothalamus were investigated to evaluate the ability of ATRA to interfere with ER mediated hypothalamic effects under dosing conditions that suppressed the EB/P primed LH surge. These endpoints included examination of changes in unoccupied ER levels and PR mRNA expression in estrogen responsive hypothalamic regions associated with regulation of LH release.

Rats were bilaterally OVX on day 0. Animals were dosed with 300 mg ATRA/kg in a 0.5% CMC suspension or an equal volume of 0.5% CMC via oral gavage between 0900 to 1000 h for five consecutive days (days 2–6). At 0900 h on days 4–6, rats were injected sc with corn oil vehicle or EB (10 μg/100 g bw) dissolved in oil. This resulted in four groups of rats (n = 6/group): (1) 0.5% CMC + OIL, (2) ATRA + OIL, (3) ATRA + EB, (4) 0.5% CMC + EB. Rats were decapitated and brains were collected, frozen on dry ice, and stored in -80^0C until sectioned.

Slide preparation. Serial coronal sections (20 μm) were cut on a 1720 Digital cryostat (Leitz Corp.) maintained at -20^0C and thaw-mounted, then immediately snap frozen on a cold metal platform at −20°C. Slides were stored desiccated at −80°C until analyzed for changes in hypothalamic ER binding and PR mRNA expression.

Changes in unoccupied ER levels following treatment with ATRA. To examine the effects of ATRA on hypothalamic estradiol binding, ER levels were assayed using an *in vitro* ER exchange assay procedure similar to previously described protocols (Walters *et al.*, 1993; Yuan *et al.*, 1995). Briefly, all slides were transferred from -80^0C freezer to a 4^0C cold room. Each slide was covered with 100 μl of TEGBD incubation buffer (10 mM Tris HCl, 1.5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 0.5 mM Bacitracin, and 0.1 mg/ml protamine sulfate, pH 7.4 @ 4°C; Sigma-Aldrich Co., St. Louis, MO) and either (1) 2 nM [^{3}H]estradiol to evaluate specific binding or (2) 2 nM [^{3}H]estradiol + 1 uM moxestrol to evaluate nonspecific binding and incubated at 30^0C for 45 min. After incubation, the sections were returned to 4^0C and rinsed in cold circulating PM rinse buffer (3.0 mM $MgCl_2$, 1.0 mM KH_2PO_4) with protamine sulfate (1 mg/ml) to remove all unbound estrogen. Tissue sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 5 min. Slides were washed (3 × 5 min in cold, circulating PM buffer with 0.1% Triton X-100 and 2 × 5 min in cold PM buffer), dipped in double deionized water and air dried overnight. Slides were placed on high resolution autoradiographic film (Amersham's 3H sensitive film) and developed for 21 days.

Evaluation of PR mRNA. *In situ* hybridization was performed to examine effects of ATRA treatment on estrogen induced expression of PR mRNA. A 320 base pair rat PR complementary DNA (cDNA) was obtained from Dr. Junzo Kato (U. Tokyo). cRNA probes generated from this cDNA recognize both the A and B form of the progesterone receptor (Hagihara *et al.*, 1992). The rat PR cDNA was linearized with *Hin*dIII and a cRNA probe was transcribed with T7 RNA polymerase in the presence of ^{35}S-UTP.

In situ hybridization for PR mRNA was carried out as previously described (Handa *et al.*, 1993). Briefly, brain sections were brought to room temperature, the sections were fixed in 10% formalin, acetylated with 0.25% acetic anhydride, dehydrated with ethanol (50–100%) washes and delipidated with chloroform. 2×10^6 cpm/μl of ^{35}S labeled riboprobe in hybridization solution of 0.1% sodium thiosulfate, 100 mM DTT, and 0.5% SDS in formamide and hybridization buffer mix (1200 nM NaCl; 20 mM Tris-HCl, pH7.5; 0.04% Denhart's; 2 mM EDTA, pH 8.0; 0.02% SSD-DNA; 0.10% Total Yeast RNA; 0.01% yeast tRNA; 20% dextran sulfate) was placed on each slide and slides were coverslipped and placed in a humidified incubator (60°C) overnight. Following hybridization, slides were consecutively washed with 2×SSC (0.3 M NaCl, 0.03 M sodium citrate; pH 7.0), treated with RNase A (20 μg/ml at 37°C for 30 min), rewashed in 0.5 and 0.1×SSC. Sections were then dehydrated with increasing concentrations of ethanol (50–100%). For autoradiography, sections were apposed to x-ray film (Hyperfilm betamax; Amersham, Lake Forest, IL) for 11 days. Hypothalamic regions sampled for PR mRNA levels were the AVPV and the MPOA.

Image analysis. For analysis of PR mRNA and unoccupied ER, film autoradiograms were used to evaluate the intensity of the hybridization signal under brightfield illumination using a computer-based imaging system using NIH Image software, version 1.61. The density of the hybridization signal within the AVPV and MPOA were measured by blind analysis using a template of fixed size. The intensity from two samples of two sequential sections per animal was subtracted from background. For evaluation of unoccupied ER, the intensity of the signal was converted to pmol/mg tissue section protein using a [^{3}H]estradiol standard curve. Specific binding was assessed by subtracting nonspecific binding from total binding. For PR mRNA levels, the intensity of the signal was converted to nCi/mg tissue section protein from a ^{14}C standard curve.

Statistics. For the LH surge studies, two-way ANOVA with repeated measures for treatment time was used to compare differences between treat-

ment group and treatment time followed by Fisher's PLSD post hoc test. Estrogen mediated effects in the hypothalamus were also evaluated with two-way ANOVA for treatment and group effects and interactions. Differences were considered significant when $p < 0.05$. Numerical values are reported as the mean ± SEM. The computer program StatView 5.0.1 (SAS Institute Inc., Cary, NC) was used for statistical analysis. Graphs were plotted with GraphPad Prism version 3.05 (GraphPad Software, San Diego, CA).

RESULTS

Effects of ATRA, or Its Metabolite DACT, on the LH Surge in Adult, Female OVX Rats

The aim of these experiments was to characterize the dose-dependent LH surge suppression after ATRA exposure and to evaluate the relative potency of DACT compared to ATRA. To date, LH suppression by ATRA has only been evaluated after priming rats with EB (Cooper *et al.*, 2000). To further characterize the ability of ATRA to suppress the LH surge, treatments with both EB and P were used to generate an LH surge. Priming with EB and P produced a peak LH surge approximately three times higher than EB priming alone (Figs. 1 and 2a). The EB/P induced LH surge was significantly suppressed by ATRA in a dose-dependent manner with 300 mg ATRA/kg/day abolishing the LH surge (Figs. 2a and 2b). Body weight loss occurred but was not significant until day 5 with the 300 mg ATRA/kg/day dose level (data not shown).

To gain insight into the ability of the chlorinated metabolites to suppress the LH surge, animals were dosed with DACT, the major chlorinated metabolite in plasma after dosing with ATRA (McMullin *et al.*, 2003). The concentration of DACT chosen was based on kinetic studies in our laboratory where 300 mg ATRA/kg/day produced a plasma AUC of DACT equivalent to dosing of 77 mg DACT/kg/day. Although to a lesser degree than ATRA, DACT significantly suppressed the total plasma LH AUC and peak LH surge levels in EB/P primed animals by 60 and 58%, respectively (Figs. 3a and 3b).

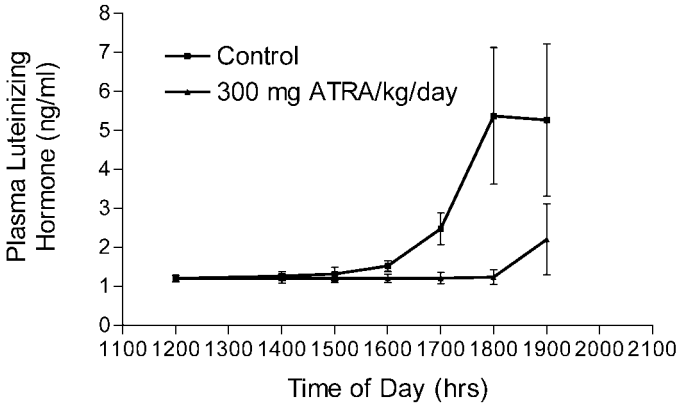

FIG. 1. Suppression of the estrogen induced LH surge by five daily doses of 300 mg ATRA/kg/day in adult ovariectomized Sprague-Dawley rats. Peak LH levels in controls and ATRA treated animals were 5.37 ± 1.76 ng/ml and 2.20 ± 0.906 ng/ml, respectively. Points and bars represent mean ± SEM.

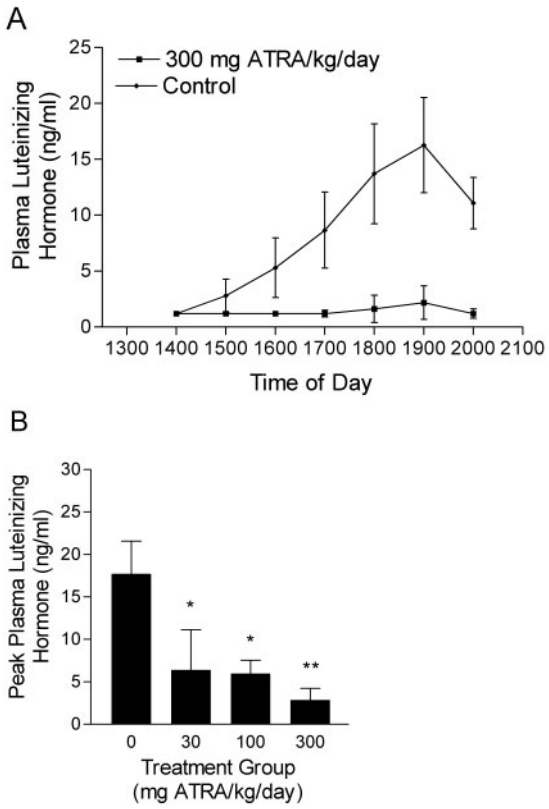

FIG. 2. Dose-dependent suppression of 300, 100, or 30 mg ATRA/kg/day for five days on the estrogen and progesterone primed LH surge in adult female ovariectomized Sprague-Dawley rats. (a) Representative time-course of the LH surge for 300 mg ATRA/kg/day and control groups. Three-hundred mg ATRA/kg/day completely blocked the LH surge. (b) ATRA suppresses peak plasma LH concentrations in a dose-dependent manner. Points and bars represent mean ± SEM. *$p < 0.05$ compared to controls; **$p < 0.001$ compared to controls.

Effect of DACT on Pituitary LH Release after GnRH Challenge

This study examined the effect of DACT on pituitary responsiveness to exogenous GnRH. DACT treatment attenuated the LH response by 47% compared to control (31.5 ± 4.8 ng/ml versus 59.4 ± 15.03 ng/ml, respectively; Fig. 4a). Total plasma LH secretion over the entire 60 min (AUC) was reduced by 37% compared to control (174.7 ± 16.2 ng/ml/min versus 274.0 ± 40.0 ng/ml/min, respectively; Fig. 4b).

The Effects of ATRA or DACT on Estradiol Binding to ERα in Vitro

Initial binding studies were performed with uterine cytosolic ER to determine the ability of ATRA to interfere with estradiol binding to this receptor. In addition, we also determined whether preincubation of ATRA with the receptor would lead to greater reduction of estradiol binding than noted on short incubations. At all time points examined, ATRA decreased [^{3}H]estradiol binding to uterine cytosol, independent of prein-

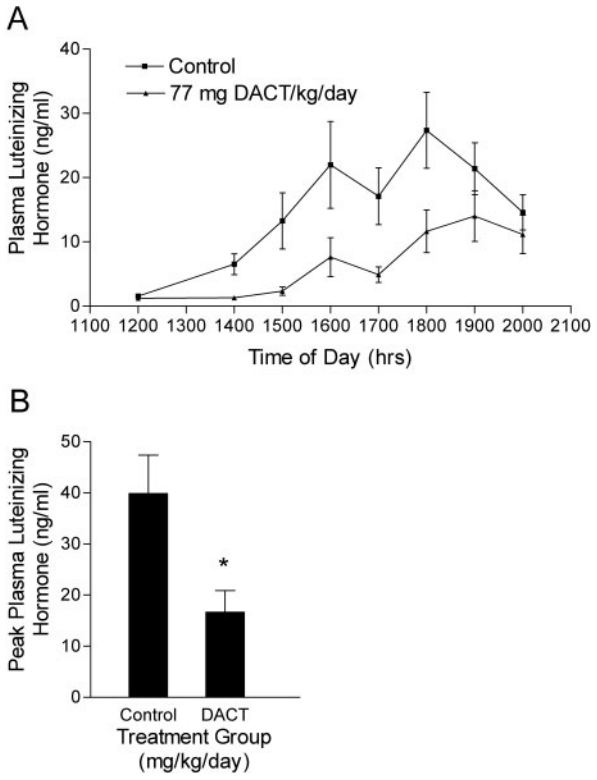

FIG. 3. Dosing with 77 mg DACT/kg/day (the equivalent AUC concentration as dosing with 300 mg ATRA/kg/day) (a) causes suppression of the amplitude of the LH surge and (b) produces a 58% decrease in the peak LH surge levels in adult female ovariectomized Sprague-Dawley rats primed with EB and P. Points and bars represent mean ± SEM. $*p < 0.05$ compared to controls.

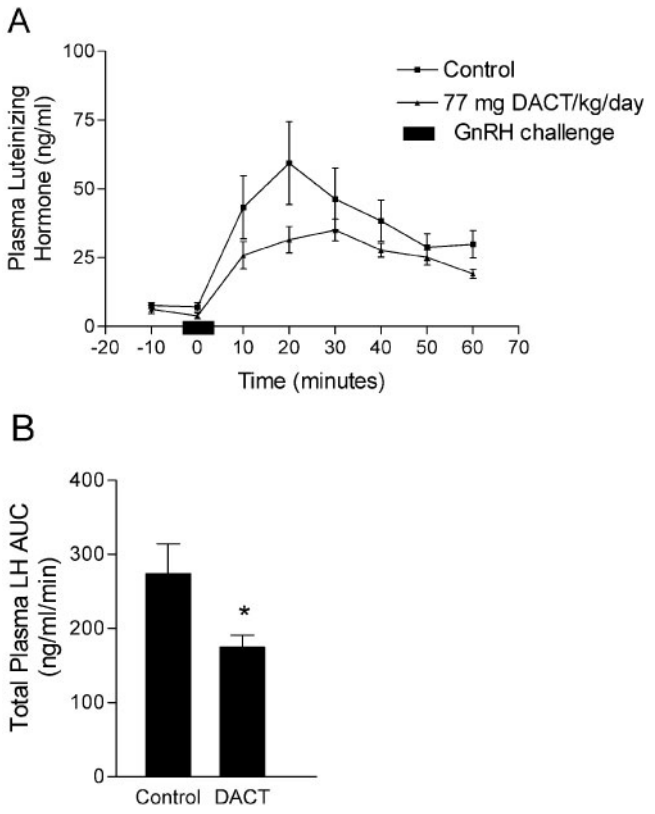

FIG. 4. DACT, under conditions which suppress the LH surge, decrease release of LH from the pituitary. (a) Plasma LH time course of DACT and control animals after iv injection of 100 ng GnRH/100 g bw. (b) Total LH secretion over 60 min, as indicated by AUC, from DACT and control animals. Points and bars represent mean ± SEM. $*p < 0.05$ compared to controls.

cubation conditions (Fig. 5). The apparent Ki of ATRA is 0.02 mmol compared to 0.13 nmol for estradiol.

To examine the direct binding of ATRA to ERα, ATRA was incubated simultaneously with [^{3}H]estradiol and *in vitro* transcribed ERα. ATRA reduced [^{3}H]estradiol binding to ERα in a dose-dependent manner (Fig. 6). Since DACT is the major chlorinated metabolite in the plasma after dosing with ATRA, the ability of DACT to reduce estradiol binding to ERα was also investigated. DACT, at the molar equivalent concentration of ATRA, had no effect on [^{3}H]estradiol binding to ERα, with an apparent Ki 10× greater than ATRA (Ki = 0.2 mmol vs. 0.02 mmol; Fig. 6).

Effects of ATRA Exposure on Estrogen-Mediated Effects in Two Estrogen Responsive Hypothalamic Nuclei

Changes in unoccupied ER levels after in vivo *treatment with ATRA.* To determine the ability of *in vivo* ATRA exposure to alter binding of estrogen to hypothalamic ER, unoccupied ER levels were evaluated in AVPV and MPOA, two hypothalamic nuclei that are responsive to estrogen and contain high levels of ER. The ligand bound (occupied) receptor can be discriminated from the unoccupied receptor by incubation conditions (Yuan *et al.*, 1995). While CMC + EB treatment significantly decreased unoccupied ER levels compared to oil controls, treatment with ATRA + EB under dosing conditions that suppress the LH surge did not alter AVPV and MPOA unoccupied ER levels compared to EB treatment group (Fig. 7).

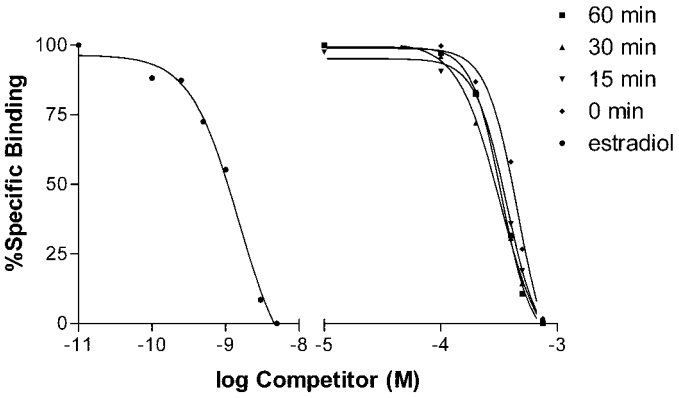

FIG. 5. Independent of *in vitro* preincubation time, ATRA inhibits binding of [^{3}H]estradiol to uterine cytosol in a dose-dependent manner. Each curve represents various preincubation times of ATRA in the cytosolic incubation mixture prior to addition of [^{3}H]estradiol.

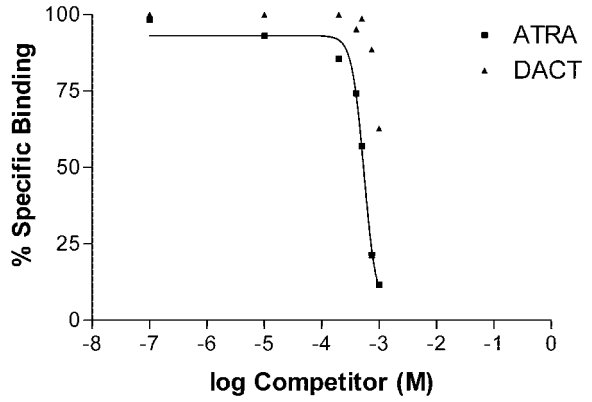

FIG. 6. ATRA and DACT were incubated simultaneously with [^{3}H]estradiol and *in vitro* transcribed ERα. ATRA reduces [^{3}H]estradiol binding to ERα in a dose-dependent manner. DACT, at the molar equivalent concentration of ATRA, has no effect on [^{3}H]estradiol binding to ERα.

Evaluation of changes in progesterone receptor mRNA expression. EB treatment significantly increased PR mRNA expression in the AVPV and MPOA. However, ATRA + EB did not alter the AVPV and MPOA PR mRNA expression compared to the EB-alone group (Fig. 8).

DISCUSSION

LH surge suppression in adult, female OVX rats due to high doses of ATRA is indicative of large-scale neuroendocrine disruption. This effect is critical in determining risk of Cl-TRI to humans. However, only limited mechanistic data are available with which to draw conclusions on the human relevance of this effect. Because ATRA is extensively metabolized, plasma (Brzezicki *et al.*, 2003) and brain (McMullin, unpublished data) Cl-TRI concentrations after ATRA treatment are largely DACT, the di-dealkylated chlorinated metabolite of ATRA. Yet, the ability of DACT to alter neuroendocrine function has not been directly evaluated. Therefore, these studies were important to investigate the mechanism by which the Cl-TRIs are acting to suppress the LH surge and to understand the significance of DACT in contributing to this effect.

ATRA and DACT Decreased the EB/P Primed LH Surge Response in Adult, Female OVX SD Rats

ATRA suppressed the EB/P LH surge in a dose-dependent fashion, almost completely abolishing the surge when administered at the highest dose for five days. Although slightly different dose regimens have been examined by others, these results are consistent with the suppression of the EB induced LH surge by ATRA as reported by Cooper *et al.* (2000). However, these studies evaluated the LH surge under conditions of estrogen priming only. While this hormonal condition may be sufficient to observe treatment differences, priming with P in addition to EB enhances the LH surge, producing conditions more realistic of the *in vivo* hormonal environment prior to the LH surge (Lee *et al.*, 1990). This observation is substantiated by our current studies where EB/P priming in OVX rats produced a peak LH surge approximately three times greater than did priming with EB alone (Figs. 1 and 2a). Additionally, these priming conditions allowed for greater sensitivity for the dose-response studies and for clearer comparisons of potency between ATRA and DACT.

As noted above, DACT is the primary CI-TRI metabolite. DACT also suppressed the LH surge in OVX, EB/P primed female SD rats (Fig. 3). The percent of peak LH suppression after dosing with DACT was only 58% while ATRA dosing at the equivalent plasma AUC completely abolished the LH surge. This may be due, in part, to the different kinetic behaviors between ATRA and DACT. One difference in kinetic behavior is the rate of clearance by phase II processes, primarily glutathione-conjugation, where the total clearance of alkyltriazines by conjugation must be almost as large as their oxidative clearance (McMullin *et al.*, 2003). Additionally, *in vivo* time-course studies indicate that direct dosing with DACT at the molar equivalent of ATRA produces peak DACT plasma

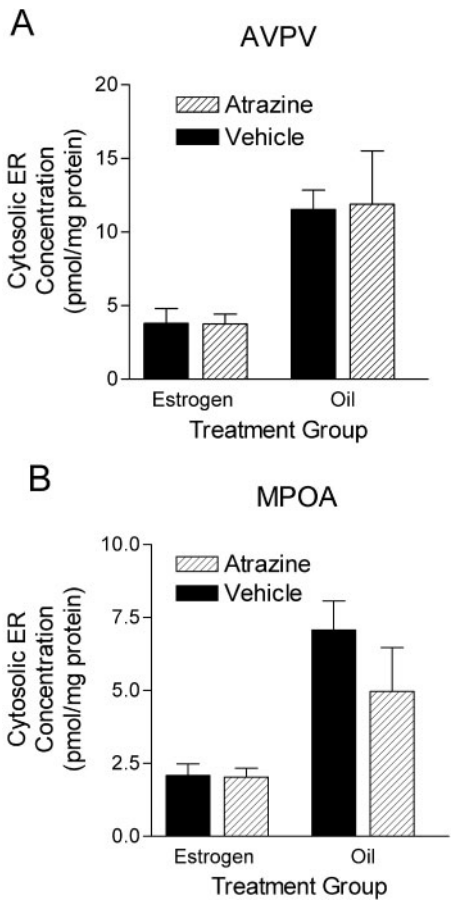

FIG. 7. Three-hundred mg ATRA/kg/day for five days does not alter levels of unoccupied ER in various estrogen responsive hypothalamic nuclei (a) anteroventroperiventricular region (AVPV) (b) medial preoptic area (MPOA) in adult ovariectomized EB primed female SD rats compared to EB primed controls. Bars represent mean ± SEM.

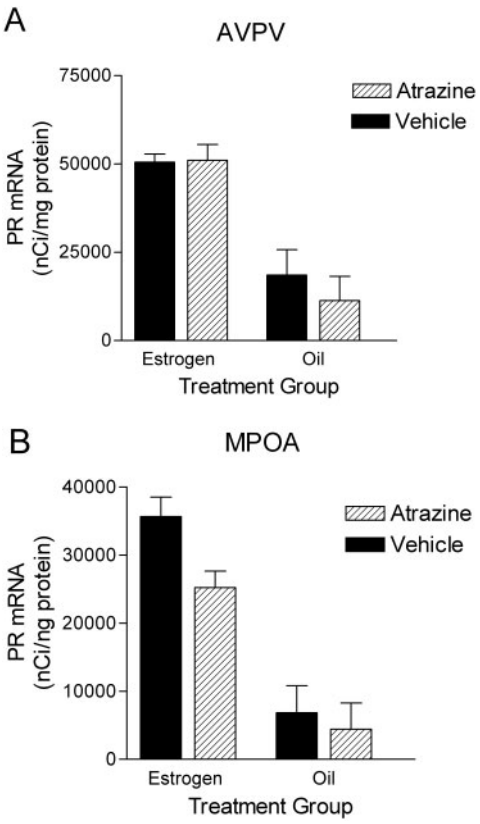

FIG. 8. In adult, ovariectomized female SD rats primed with EB, five daily doses of 300 mg ATRA/kg/day does not alter estrogen induced PR mRNA upregulation in various estrogen responsive hypothalamic nuclei compared to controls. (a) anteroventroperiventricular region (AVPV) (b) medial preoptic area (MPOA) compared to EB primed controls. Bars represent mean ± SEM.

concentrations approximately four times greater than when dosing with ATRA, also suggesting different rates of absorption and clearance (Brzezicki *et al.*, 2003). Due to these differences, further work will be required to assess relative potency of these compounds based on some consistent measure of target tissue dose or at least a measure of plasma concentrations of triazines and their metabolites.

DACT Decreased Pituitary LH Release after GnRH Challenge

In order to further examine the site of action of the Cl-TRIs, pituitary function was evaluated using the EB/P priming model and dosing conditions of DACT that suppress the LH surge in our studies. DACT significantly decreased the peak LH concentrations and the overall output of pituitary LH release compared to controls. This behavior indicates that under these conditions, DACT decreases pituitary and/or hypothalamic function.

A previous study indicated that the pituitaries of OVX, estrogen primed female Long Evans rats receiving three daily doses of 300 mg ATRA/kg/day were capable of releasing normal levels of LH after exogenous GnRH administration (Cooper *et al.*, 2000). The observed differences between ATRA and DACT may be due to differences in experimental design. For instance, in our study, animals were primed with EB and P instead of EB alone and animals were challenged with 50% greater GnRH. EB/P priming elevated LH levels 10× greater than EB priming alone. Cooper and coworkers determined that, in the presence of exogenous GnRH, the pituitaries of ATRA treated animals are capable of releasing LH. However, our study indicates that, while the pituitaries of DACT treated animals are able to release LH in response to a GnRH challenge, the response is significantly reduced compared to controls. The differences between ATRA and DACT on pituitary release of LH after GnRH administration may also be due to differences in kinetic behaviors of the Cl-TRI. Such differences could lead to greater exposure of the pituitary and/or hypothalamus to DACT in DACT treated animals than from ATRA treatment, resulting in altered pituitary function.

While it may be possible for all Cl-TRI to elicit neuroendocrine effects at high doses, our kinetic results (Brzezicki *et al.*, 2003) indicate that DACT, not ATRA itself, is most likely responsible for disruption leading to suppression of the LH surge. The possible role of glutathione conjugates formed from ATRA and the two mono-de-alkylated metabolites remains unknown.

ATRA and DACT Alter Estrogen Binding to ER in Vitro *but Not* in Vivo

To investigate the mechanism by which exposure to ATRA or DACT leads to altered LH release, we examined changes in ER activity *in vitro* after ATRA and DACT treatment and modulation of estrogen responses in the hypothalamus of ATRA exposed animals. *In vitro*, ATRA reduced [^{3}H]estradiol binding to uterine cytosolic ER (Fig. 5) and reduced binding of [^{3}H]estradiol to ERα in a dose- dependent manner (Fig. 6). In contrast to a previous report (Tennant *et al.*, 1994a), there was no effect of preincubation in determining the extent of reduction in [^{3}H]estradiol binding caused by ATRA. Perhaps these differences may be attributed to the different range of concentrations and incubation times used.

Due to the rapid metabolism of ATRA to the mono and di-dealkylated chlorinated metabolites, a single dose of 300 mg ATRA/kg is expected to produce an *in vivo* peak plasma concentration of ATRA of only 0.50 mg/l (McMullin *et al.*, 2003). This concentration is approximately 400-fold lower than the highest dose of ATRA used in the *in vitro* binding studies (216 mg/l), making it unlikely that brain concentrations of ATRA after *in vivo* dosing would approach the high end of *in vitro* doses used in this study. However, the range of *in vitro* concentrations in the binding studies with DACT (0–146 mg/l) are more similar to the estimated *in vivo* peak plasma concentrations of DACT after dosing with ATRA (28 mg/l) or dosing directly with DACT at the molar equivalent to ATRA (80 mg/l). Even at these high concentrations, DACT does not

inhibit binding of [^{3}H]estradiol to ERα. Tennant *et al.* (1994b) showed that ATRA and DACT were capable of binding ER in uterine cytosol in a binding study when allowed to preincubate for 30 min prior to addition of [^{3}H]estradiol. Therefore, DACT, unlike ATRA, may require preincubation to bind to purified ERα. In conclusion, high concentrations of ATRA, but not DACT, can inhibit [^{3}H]estradiol binding to ERα *in vitro*. The steepness of the binding curve of ATRA suggests that ATRA likely binds ERα and inhibits binding of estradiol through noncompetitive, rather than competitive binding and should be further investigated.

In vitro findings and previous studies with ATRA indicated that a possible direct anti-estrogenic effect of ATRA may occur *in vivo* to produce the suppression of the LH surge (Tennant *et al.*, 1994a,b). However, many known anti-estrogens are tissue specific in their ability to act as either an estrogen agonist or antagonist. Therefore, our studies, which evaluated ER activity in the target tissue after ATRA treatment, were significant in determining the relevance of ATRA's anti-estrogenic properties to suppression of the LH surge.

EB treatment of OVX rats causes significant increases in PR mRNA expression in various estrogen responsive hypothalamic nuclei (Chappell and Levine, 2000; Shughrue *et al.*, 1997; Simerly *et al.*, 1996). This stimulation is a direct effect of estradiol on PR gene expression and estrogen antagonists such as tamoxifen and raloxifene decrease PR mRNA expression in the hypothalamus (Shughrue *et al.*, 1997). ATRA, however, did not alter the estradiol induced PR mRNA expression in the AVPV and MPOA of the hypothalamus. Additionally, ATRA did not alter the binding of estrogen to ER in these same hypothalamic regions. It is worth noting that compounds that have an *in vitro* affinity and potency similar to estradiol, such as tamoxifen and raloxifene, require *in vivo* concentrations 100 to 1000-fold higher than *in vitro* to produce an anti-estrogenic effect on PR mRNA in the hypothalamus (Shughrue *et al.*, 1997). Similarly, ATRA and DACT may be binding ER *in vivo*, but their anti-estrogenic activity is not potent enough to compete with estradiol under *in vivo* conditions.

Despite evidence that ATRA and DACT may possess weak anti-estrogenic properties *in vitro*, our studies indicate that the decreased responsiveness of the hypothalamus and/or pituitary to GnRH challenge and the consequent suppression of the LH surge after treatment with high doses of ATRA and DACT are most likely not due to estrogen antagonist activity. These results also suggest that DACT is more likely to be responsible for endocrine disruption leading to suppression of the LH surge than ATRA. Although the LH surge is largely regulated by binding of estradiol to ERα, there are multiple neurotransmitter and neuroendocrine signals that directly and indirectly modulate LH release from the pituitary. For example, GnRH neurons play a central role in reproductive cycling and constitute the final common pathway of signaling from the brain, receiving a multitude of upstream stimulatory and inhibitory influences from other neurons. These signals include dopamine, glutamate, estradiol, nitric oxide, glucocorticoids, neuropeptide Y, leukotrienes, and many others. Depending on the timing, some of these influences are both stimulatory and inhibitory (Kalra and Kalra, 1983). It is an integration of these upstream signals that influence the patterns of GnRH release from the neurons into the median eminence and ultimately, the release of LH from the anterior pituitary. It is possible that these are targets for DACT; however, further investigation will be required to elucidate the importance of these interactions.

These studies also highlight the importance of proper design of *in vitro* mechanistic studies to study pituitary and hypothalamic effects of Cl-TRIs. Such studies should consider the treatment regimen in the context of the *in vivo* kinetic behavior of ATRA. While it is challenging to design a proper *in vitro* study that directly compares with the *in vivo* scenario, direct treatment with DACT, or potentially other metabolites, is essential for allowing more appropriate interpretation between *in vitro* and *in vivo* effects. *In vivo* treatment with DACT eliminates many variables due to metabolism, allowing for a more direct interpretation of the mechanism by which chlorotriazines are altering the LH surge and aiding in more appropriate risk assessment.

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