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Octamethylcyclotetrasiloxane exhibits estrogenic activity in mice via ERα

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Abstract

Octamethylcyclotetrasiloxane (D4) is a low molecular weight cyclic silicone used in the synthesis of larger silicone polymers and in the formulation of a variety of personal care products. The effects of oral D4 exposure in mice on serum estradiol levels, uterine wet weight, and uterine peroxidase activity were investigated. Additionally, in vitro estrogen receptor binding activity was evaluated. Serum estradiol levels decreased in a dose-dependent manner after exposure to 100 mg/kg to 1000 mg/kg D4. Studies with adrenalectomized animals demonstrated that the decreased serum estradiol levels were not due to elevated serum corticosterone levels. Uterine wet weights in ovariectomized mice were significantly increased in a dose-dependent manner by exposure to 250–1000 mg of D4/kg, but not by exposure to other silicone compounds tested (hexamethylcyclotrisiloxane, decamethylcyclopentasiloxane, decamethyltetrasiloxane, and octaphenylcyclotetrasiloxane). Uterine peroxidase activity, a marker for estrogenic activity, was also significantly increased in D4-exposed mice, but not in mice exposed to the other siloxanes. Pretreating mice with the estrogen receptor antagonist ICI 182,780 completely blocked the D4-induced increase in uterine weight, and ovariectomized estrogen receptor-α knockout mice showed no increases in uterine weights when orally exposed to D4 or estradiol. In an in vitro estrogen receptor binding assay, D4 showed significant competition with ^{3}H-estradiol for binding to estrogen receptor-α, but not estrogen receptor-β. The data presented here indicate that D4 has weak estrogenic activity, and that these effects are mediated through estrogen receptor-α.

Keywords: Octamethylcyclotetrasiloxane; Estrogenic activity; Uterotrophic assays; Estrogen receptors; ERKO; ICI 182,780

Introduction

Octamethylcyclotetrasiloxane (D4), a low molecular weight (m.w.) cyclic siloxane (m.w. 296) is used in the manufacturing of many high molecular weight silicone polymers including those used for medical devices. D4 and other siloxane-based compounds have been incorporated into the manufacture of many personal care compounds (Burns-Naas et al., 2002) resulting in potential exposure to D4 by the dermal, inhalation, and oral routes. The pharmacokinetics of D4 have been well studied (Plotzke et al., 2000; Andersen et al., 2001; Sarangapaini et al., 2003). D4 has been shown to be highly lipophilic, metabolized mainly by the liver, and eliminated by exhalation and excretion with the rates being dependant on the route of exposure. PK modeling has been used to estimate that approximately 2% of delivered D4 remains in fat deposits in the body (Sarangapaini et al., 2003) and D4 has been recovered from the ovaries of exposed rats (Plotzke et al., 2000).

In recent years, some reports have indicated that D4 may have deleterious effects on biological systems. In animal studies, D4 exposure resulted in alterations in immune responses (LeVier et al., 1995; Wilson, 1996), changes in

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organ weights (Meeks et al., 1995; Wilson, 1996), induction of hepatic drug metabolizing enzymes (McKim et al., 1998; Zhang et al., 2000), and an adverse impact on reproductive health (Mast et al., 1997; Stump et al., 2000). One study reported that D4 exhibited estrogenic activity in the classic uterotrophic assay (McKim et al., 2001); however, the mechanism by which D4 exerts uterotrophic effects has not been investigated. The objective of the current studies was to further characterize the estrogenic potential of D4 and to investigate the mechanism by which D4 induces estrogenic activity. To this end, the effects of D4 on serum estradiol (E2) level, uterine weight, and uterine peroxidase activity were determined in mice. The estrogen receptor (ER) antagonist ICI 182,780 and estrogen receptor-α knockout mice (αERKO) were used to examine the role of the ER in the estrogenic activity of D4, and in vitro studies directly investigated the extent to which D4 directly binds the ligand-binding site of ERα and ERβ.

Methods

Animals. Female estrogen receptor-α knockout (αERKO) mice (Korach, 2000) were provided from the NIEHS breeding facility under an approved animal protocol. Wild-type control mice with the same genetic background (129/J/C57BL/6J) as the αERKO mice and female B6C3F1 mice were purchased from Taconic, Germantown, NY. Mice were 5–6 weeks old upon arrival and were allowed to adapt to their environment for 1 week prior to the experiments. The mice were provided standard diet and tap water ad libitum. The animals were maintained under conditions specified within NIH guidelines. Animal rooms were maintained at 18–26°C and 40–70% relative humidity with light/dark cycles of 12-h intervals.

Adrenalectomized (ADX) and sham-operated mice were single-housed, and ADX mice were given 1% saline-1% sucrose solution supplemented with 30 μg/ml corticosterone for survival (Le, 1997). Prior to the experiments, ovariectomized (OVX) mice were kept for 2 weeks post surgery to allow endogenous hormones to decline.

Chemicals. Octamethylcyclotetrasiloxane (D4) was obtained from Dow Corning Corporation (Midland, MI). Hexamethylcyclotrisiloxane (D3), decamethylcyclopentasiloxane (D5), decamethyltetrasiloxane (L4), and octaphenylcyclotetrasiloxane (phenyl-D4) were purchased from Gelest, Inc. (Tullytown, PA). For in vivo studies, dilutions of siloxanes were prepared in corn oil. 17β-Estradiol (E2) was purchased from Sigma Chemical (St. Louis, MO) and diluted in corn oil/1% ethanol. ICI 182,780 (ICI) was purchased from Tocris (Ballwin, MO) and diluted in corn oil/5% ethanol. [^{3}H]-E2 (S.A. 89 Ci/mmol) was purchased from Amersham (Piscataway, NJ).

Exposure. In studies where serum E2 and corticosterone levels were determined, mice were dosed with 1000 mg/kg D4 orally, unless otherwise indicated, for 7 consecutive days. Twenty-four hours after the last dose, mice were euthanized, and sera were collected for the determination of E2 and corticosterone levels.

In the uterotrophic assays, mice were dosed with test solutions for three consecutive days. Siloxanes (at dosage of 1000 mg/kg unless otherwise indicated) were given orally, and E2 (10 μg/kg) was given by subcutaneous injection. In studies where the ER antagonist was used, ICI (20 mg/kg) was given by subcutaneous injection 30 min prior to dosing with D4 or E2. Twenty-four hours after the last dose, mice were euthanized and uteri were excised just above the junction with the cervix and at the junction of the uterine horns with the ovaries. Uteri were trimmed free of fat and weighed. Uteri were then flash-frozen in liquid nitrogen and stored at −80°C until further handling.

Serum E2 and cortocosterone assays. Serum E2 levels were determined using the Estradiol ELISA kit (American Laboratory Products Co., Windham, NH). The assay was performed according to the manufacturer's instructions, including ether extraction of steroids. Briefly, E2 standards or extracted serum samples were dispensed into appropriate wells in 96-well microtiter plates coated with anti-estradiol serum. Then, E2-conjugate (E2 conjugated to horseradish peroxidase) was added into each well. After incubation for 2 h at room temperature, the wells were washed. Substrate solution was added to each well and incubated for 15 min at room temperature. The enzymatic reaction was stopped and the absorbance was determined at 450 nm with a microplate reader.

Serum corticosterone levels were determined by Coat-A-Count corticosterone RIA kit (Diagnostic Product Corporation, Los Angeles, CA). Briefly, serum samples were added to the corticosterone antibody-coated tubes. ^{125}I-labeled corticosterone was then added. After a 2-h incubation at room temperature, the tubes were decanted and counted for 1 min in a gamma counter.

Uterine peroxidase assay. E2-inducible uterine peroxidase was extracted, and its activity was measured as described by Lyttle and DeSombre (1977). Briefly, each frozen uterus was quickly thawed and placed in ice-cold 10 mM Tris (pH 7.2) to achieve 25 mg tissue/ml Tris. Samples were disrupted using a Polytron PT 10ST (setting 5–6) for 10 s in an ice bath, then centrifuged 45 min at 39,000 × *g*, 2°C. The supernatants were discarded and peroxidase was extracted from the pellets by dispersing them in 10 mM Tris-HCl (pH 7.2), 0.5 M $CaCl_2$ using a Polytron as described above. The 0.5 M $CaCl_2$ extracts were centrifuged 45 min at 39,000 × *g*, 2°C, and supernatants were assayed for peroxidase activity by measuring the rate of guaiacol oxidation at 25°C as described (Lyttle and DeSombre, 1977), except the guaiacol concentration was increased to 26 mM. The higher concen-

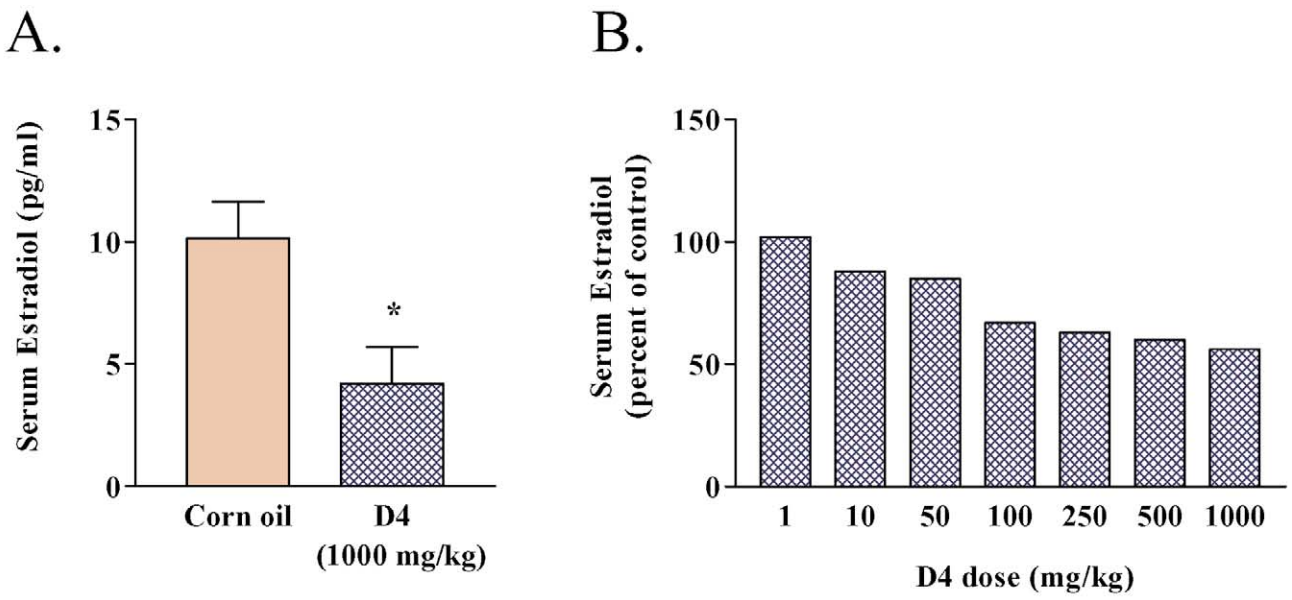

Fig. 1. Effect of D4 exposure on serum E2 levels. Female B6C3F1 mice ($N \geq 6$/group) were dosed orally with 0.1 ml/10 g of body weight of corn oil (control) or indicated doses of D4 for 7 consecutive days. Twenty-four hours after the last dose, mice were euthanized and sera were collected for the determination of E2 by enzyme-linked immunosorbent assay. (A) Results are presented as mean pg/ml E2 ± SE; *$P < 0.05$, compared to control (oil). (B) Results are expressed as percentage of control.

tration of guaiacol produced more reproducible results and was linear with enzyme extract over the ranges tested.

ER binding assay. ER competition binding studies were performed using purified human recombinant ERα and ERβ (Panvera, Madison, WI). Reactions containing 1 pmol ER, 1 pmol [^{3}H]-E2, and indicated concentrations of competing unlabeled E2 or D4 in 10 mM Tris, pH 7.5, 10% glycerol, 2 mM dithiothreitol, and 1 mg/ml bovine serum albumin were incubated in a 37°C water bath for 30 min. ER with bound [^{3}H]-E2 was separated from free [^{3}H]-E2 by the addition of hydroxylapatite and repeated centrifugation and washing. Liquid scintillation counting was used to determine the amount of [^{3}H]-E2 bound to ER.

Statistical analysis. Unpaired *t* tests were performed on data between two groups. When multiple comparison were used, a one-way analysis of variance was performed, and when significant differences ($P < 0.05$) occurred the Dunnett's *t* test was performed.

Results

D4 significantly reduced serum estradiol levels

Initial studies were conducted to determine the extent to which orally administered D4 might alter serum E2 levels. The initial dose of D4 administered (1000 mg/kg) was chosen based on concentrations demonstrated to induce immunosuppressive effects in the absence of body weight loss (Wilson, 1996). Following 7 days of exposure to this dose of D4, serum estradiol levels were reduced ~50% (Fig. 1A). In subsequent studies, the suppression of E2 levels by D4 was shown to be reproducible and dose dependent (Fig. 1B).

Oral administration of D4 to mice and rats has been shown to increase serum corticosterone levels (Wilson, 1996). To investigate the role of corticosterone in the D4-mediated reduction of serum E2, studies were conducted using adrenalectomized (ADX) mice. As previously reported, D4 increased corticosterone levels in intact and sham-operated mice; however, in ADX mice serum corticosterone levels were not elevated following exposure to 1000 mg/kg D4 (Fig. 2A). Levels of serum E2 in ADX mice were similar to the levels observed in intact and sham-operated mice, and in all of these animals D4 exposure resulted in reduced serum E2 levels (Fig. 2B). The absence of a correlation between levels of serum corticosterone and E2 (Fig. 2) demonstrates that the D4-induced decrease in serum E2 levels is independent of serum corticosterone levels.

Exposure to D4, but not other siloxanes, significantly increased uterine weights and uterine peroxidase activity in OVX mice

A series of low molecular weight siloxanes have been shown to modulate serum corticosterone levels, stomach weights (Wilson, 1996), and hepatic drug metabolizing en-

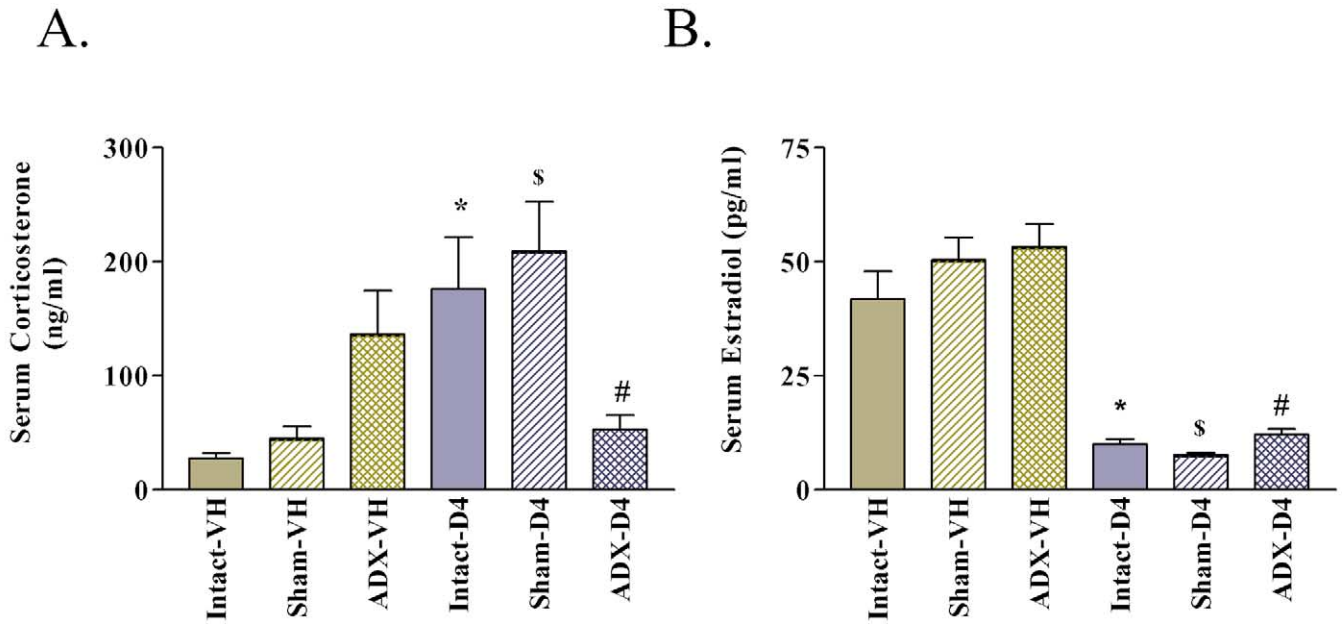

Fig. 2. Serum corticosterone and E2 levels in adrenalectomized (ADX) mice following D4 exposure. Intact, ADX or sham-operated female B6C3F1 mice ($N = 8$/group) were orally dosed with corn oil (VH) or 1000 mg/kg D4 for 7 days. Twenty-four hours after the last dose, mice were euthanized and sera were collected for the determination of corticosterone and E2; serum corticosterone levels (A) were determined by radioimmunoassay and serum E2 levels (B) were determined by enzyme-linked immunosorbent assay, as described. Bars represent the mean ± SE, $^{*}P < 0.01$, compared to Intact-VH; $^{\$}P < 0.01$, compared to Sham-VH; $^{\#}P < 0.05$, compared to ADX-VH.

zymes (McKim et al., 1999; Zhang et al., 2000). Therefore, a panel of silicone compounds was tested for estrogenic activity using an uterotrophic assay and an uterine peroxidase assay. At a concentration of 1000 mg/kg, D4 was the only siloxane tested that induced a significant increase in uterine weight (Fig. 3A) or uterine peroxidase activity (Fig.

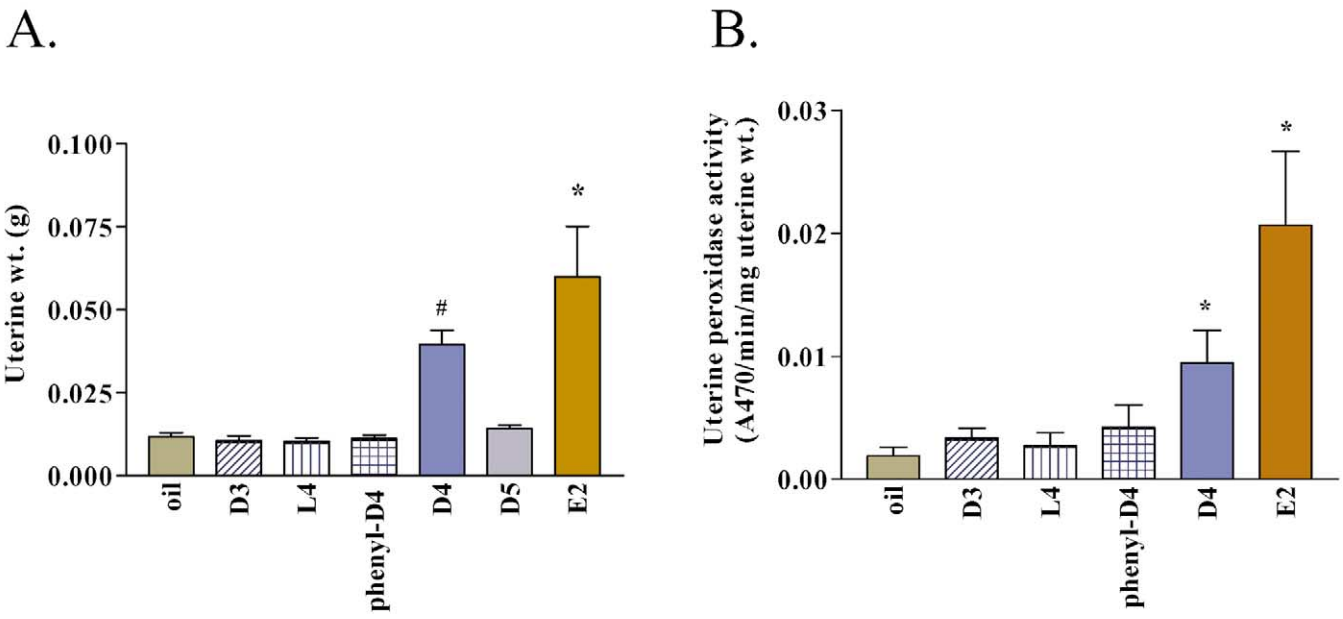

Fig. 3. Effects of various siloxanes on uterus weights and uterine peroxidase activity in ovariectomized mice. Ovariectomized female B6C3F1 mice ($N = 5$) were dosed with the indicated test solutions for 3 days. Corn oil (oil) and siloxanes (D3, L4, phenyl-D4, D4, or D5) 1000 mg/kg were given orally, and E2 (10 μg/kg) was given by subcutaneous injection. Twenty-four hours after the last dose, mice were euthanized and uteri were removed and weighed (A); peroxidase was extracted from all samples except those exposed to D5 and activity was assayed (B), as described. Peroxidase activity was expressed as the rate of guaiacol oxidation (A470/min) and normalized to milligrams of uterine weight. Bars represent the mean ± SE, $^{\#}P < 0.01$, compared to control (oil); $^{*}P < 0.001$, compared to control (oil).

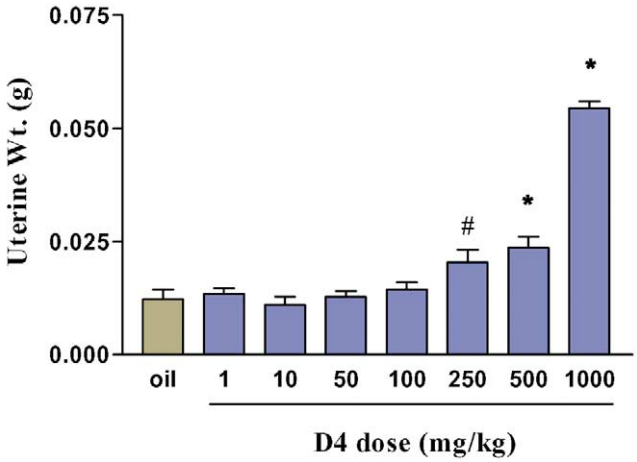

Fig. 4. Effects of D4 exposure on uterus weight in ovariectomized mice. Ovariectomized female B6C3F1 mice ($N = 5$) were dosed orally with corn oil or the indicated doses of D4 for 3 days. Twenty-four hours after the last dose, mice were euthanized and uteri were removed and weighed. Bars represent the mean ± SE, $^{\#}P < 0.05$, compared to control (oil); $^{*}P < 0.01$, compared to control (oil).

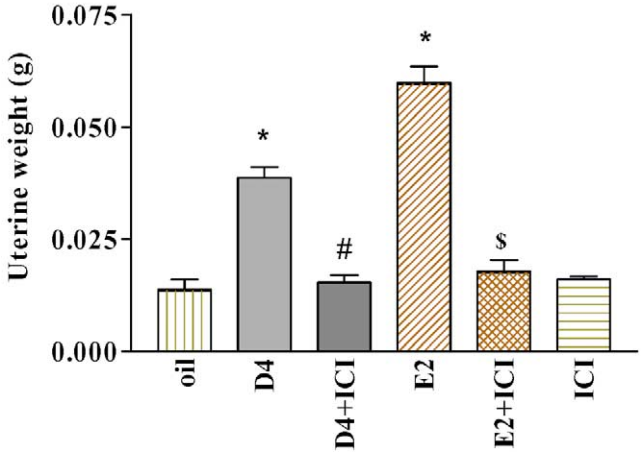

Fig. 5. Effect of ICI 182,780 on uterus weights in ovarectomized mice. Ovaricetomized female B6C3F1 mice ($N = 5$) were pretreated with estrogen receptor antagonist ICI 182,780 (20 mg/kg) by subcutaneous injection 30 min prior to dosing with corn oil, D4 (1000 mg/kg, po), or E2 (10 μg/kg, sc injection) for 3 days or were dosed with corn oil, D4, or E2 without ICI 182,780 pretreatment. Twenty-four hours after the last dose, mice were euthanized and uteri were removed and weighed. Bars represent the mean ± SE, $^{*}P < 0.001$, compared to oil. $^{\#}P < 0.001$, compared to D4. $^{\$}P < 0.001$, compared to E2.

3B). A dose-response study showed that D4 significantly increased uterine weights at doses ranging from 250 to 1000 mg/kg (Fig. 4).

ERα mediates the estrogenic effects of D4 in mouse uterus

To test the hypothesis that the uterotrophic effect of D4 is mediated through the ER, the uterotrophic assay was repeated in mice pretreated with the ER antagonist ICI 182,780. As in the previous studies, 1000 mg/kg D4 orally induced an approximate 3-fold increase in uterine weights in OVX mice. Positive control animals exposed to E2 (10 μg/kg) showed an approximate 4-fold increase in uterine weights. Pretreatment with ICI 182,780 completely blocked both the D4- and E2-induced increases in uterine weights (Fig. 5); ICI 182,780 exposure alone had no effect on uterine weight.

Ovariectomized αERKO mice and their OVX wild-type controls were used in an uterotrophic assay as one means of investigating the role of ERα in mediating the estrogenic effects of D4 in the mouse uterus. As in other uterotrophic assays, the mice were exposed to vehicle (corn oil), 250–1000 mg/kg D4, or to 10 μg/kg E2. As shown in Fig. 6, the increases in uterine weights induced by exposure to D4 or E2 in the OVX wild-type control mice were absent in exposed αERKO mice.

To directly investigate the potential of D4 to interact with the ligand binding domain of ERα and ERβ, in vitro ER binding assays were conducted using recombinant human ERα and ERβ. At 4×10^{-5} M, D4 significantly competed with [^{3}H]-E2 for binding ERα (Fig. 7). In contrast, D4 did not significantly compete with [^{3}H]-E2 for binding ERβ at even the highest concentrations tested (data not shown).

Discussion

Recent evidence that D4 might adversely impact reproductive health (Mast et al., 1997; Stump et al., 2000) and exhibit xenoestrogenic activity (McKim et al., 2001)

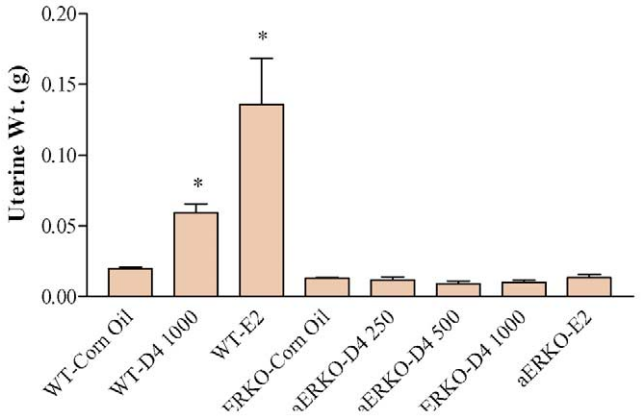

Fig. 6. Effect of D4 exposure on uterine weights in ovariectomized estrogen receptor-α knockout (αERKO) and wild-type (WT) control mice. Ovariectomized αERKO mice ($N = 5$) were dosed with corn oil, D4 (250, 500, or 1000 mg/kg, po), or E2 (10 μg/kg, sc) for 3 days and wild-type control mice ($N = 5$) were dosed with corn oil, or 1000 mg/kg D4, orally for 3 days. Twenty-four hours after the last dose, mice were euthanized and uteri were removed and weighed. Bars represent the mean ± SE, $^{*}P <$ 0.001, compared to corn oil exposed group for wild-type or αERKO mice.

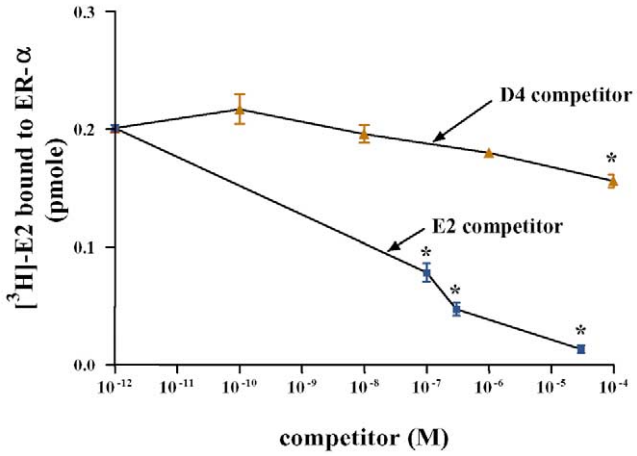

Fig. 7. D4 competition binding assay with estrogen receptor-α (ERα). An ER binding assay was performed using purified human recombinant ERα as described in the materials and methods section. Indicated concentrations of D4 (triangles) or unlabeled E2 (squares) were added to the assay as competitors with [^{3}H]-E2. Points represent the mean pmol $\pm$ SE of [^{3}H]-E2 bound to ER in the presence of the designated concentrations of each inhibitor. Samples were run in triplicates. *$P < 0.05$, compared to control (without competitor).

prompted the current studies. The goal of these studies was to better define the mechanism by which D4 alters estrogen-regulated processes in animals. To this end, a number of studies were carried out from evaluating serum estradiol levels to investigating the potential for D4 to directly bind estrogen receptors.

Estradiol levels were found to be dose-dependently decreased in D4-exposed mice. It is well known that stress induced activation of the hypothalamic-pituitary-adrenal axis may be associated with a suppression of the hypothalamic-pituitary-gonadal axis (Rabin et al., 1990). Given that earlier studies in this laboratory demonstrated an increase in corticosterone levels in D4-exposed animals, the relationship between corticosterone and estradiol levels following D4 exposure was investigated using ADX mice. The decreased levels of E2 in the absence of an elevation of corticosteroid in D4-exposed ADX mice demonstrated that the modulation of E2 by D4 could not be attributed solely to elevated serum corticosteroid levels.

Another possible mechanism relates to the effects of D4 exposure on metabolism of E2. E2 is metabolized by hydroxylation followed by glucuronidation or sulfation (Briggs and Brotherton, 1970). Although studies to measure the enzyme activity of the specific metabolic pathways of estradiol in D4-exposed animals have not been conducted, McKim et al. (1998) showed that inhalation exposure to D4 induces a spectrum of liver metabolic enzymes. These include the phase I enzymes CYP1A, CYP2B, CYP3A, and epoxide hydrolase, which are known to participate in the metabolism of estrogens. Although enzyme levels were not measured, earlier studies in our laboratory (Wilson, 1996) demonstrated liver weight increases and hepatic hypertrophy following D4 exposure by the oral route. It is hypothesized that the decrease in estradiol levels observed in these studies is mediated in part by enhanced metabolism.

The decrease in estradiol levels in D4-exposed mice did not correlate with results of the uterotropic assay. McKim et al. (2001) demonstrated weak estrogenic effects of D4 in immature Sprague-Dawley and Fischer 344 rats using a uterotrophic assay and evaluation of uterine epithelial cell height. D4 induced dose-responsive estrogenic effects in both of these strains of rats at concentrations ranging from 250 to 1000 mg/kg administered by the oral route. In the studies presented here, similar D4 doses induced a positive response as measured by the uterotropic and uterine peroxidase assays in OVX B6C3F1 mice. Contrary to the uterotropic effects of D4, the linear siloxanes have not been shown to demonstrate estrogenic activity. McKim et al. (2001) showed no estrogenic effect in Sprague-Dawley rats exposed to hexamethyldisiloxane at concentrations as high as 1200 mg/kg per day. In the studies reported here, decamethyltetrasiloxane also failed to induce estrogenic effects. Additionally, other cyclic siloxanes tested, hexamethylcyclotrisiloxane, decamethylcyclopentasiloxane, and octaphenylcyclotetrasiloxane, were negative in the uterotrophic assays.

D4 may reduce serum E2 by exerting an E2-like effect on the hypophalamic-pituitary-gonadal axis and feedback inhibiting E2 production. Although luteinizing hormone (LH) levels were not measured in these studies, in a previous report by Dalu et al. (2002) an attenuation of the LH surge on the day of proestrus was observed in a percentage of rats exposed to D4 by inhalation. Most xenoestrogens exhibit estrogenic (or anti-estrogenic) activity by directly interacting with ER. There are two main forms of ER, ERα and ERβ, which not only exhibit different tissue distributions (Enmark et al., 1997; Kuiper et al., 1997), but also can elicit different biological effects upon binding various ligands (Paech et al., 1997). In addition, some compounds are thought to activate ER by ligand binding-independent mechanisms. For example, epidermal growth factor is believed to activate ER by inducing ligand-independent phosphorylation (Bunone et al., 1996; Hafner, et al., 1996; Kato et al., 1995). The estrogenic organochlorine compounds *o,p*-DDT and β-hexachlorine hexane, are potent activators of protein tyrosine kinase in ER-positive breast cancer cells by a mechanism that appears to be ER independent (Enan and Matsumura, 1998).

While the uterotrophic effects of E2 are known to be mediated by the ER (Cooke et al., 1997; Orimo et al., 1999) the role of ER in the uterotrophic response elicited by D4 had not been established. Because the potent ER antagonist ICI 182,780 has been shown to completely block the uterotrophic effects of E2 (Howell et al., 2000; Wade et al., 1993; Wakeling et al., 1991), this antagonist was used to determine if ER was important in mediating the estrogenic effects of D4 on the mouse uterus. Pretreating mice with ICI 182,780 abolished the D4-induced, as well as the E2-in-

duced, increases in uterus weight, indicating ER is important in mediating the estrogenic effects of D4. Both ERα and ERβ are expressed in uterus tissue (Enmark et al., 1997; Kuiper et al., 1997), and αERKO mice were used to investigate the involvement of ERα in mediating the estrogenic effect of D4. In uterotrophic assays, αERKO mice have been used to show the importance of this ER isoform in mediating the estrogenic effects of compounds such as estradiol, hydroxy-tamoxifen, and diethylstilbestrol (Couse and Korach, 1999). Neither D4 nor E2 elicited a significant increase in uterus weight in OVX αERKO mice, implicating ERα in mediating the estrogenic activity of D4. Furthermore, ERα binding studies demonstrated that D4 competed with [^{3}H]-E2 binding ERα, although D4 is a weak E2 competitor. In contrast, D4 did not significantly compete with [^{3}H]-E2 binding ERβ.

In summary, these studies demonstrated that D4 elicits estrogenic activity in the uterus by an ERα-dependent mechanism that may result in part through direct receptor binding. Furthermore, these studies revealed that D4 reduces serum E2 levels in a concentration-dependent manner in intact mice. Thus, the stimulatory effect of D4 on uterus weight and on uterus peroxidase activity cannot be attributed to D4-induced increases in E2 production. The observations that D4 can both mimic E2 in some uterine responses and decrease serum E2 levels indicates that this commonly used agent may have multiple, complex effects on E2-regulated processes.

Acknowledgments

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