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2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN (TCDD) EFFECTS ON HEPATIC MICROSOMAL STEROID METABOLISM AND SERUM ESTRADIOL OF PREGNANT RATS*

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Abstract—Experiments were conducted to evaluate the effects of administration of low, but fetotoxic quantities of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) during pregnancy on steroid metabolism in liver microsomes. Oral administration of 1 $\mu g \cdot kg^{-1} \cdot day^{-1}$ of TCDD to pregnant rats on days 7–19 of gestation reduced maternal weight gain during pregnancy. Analysis of litters on day 20 showed that fetuses from TCDD-treated dams had a 66% incidence of visceral lesions characterized by intestinal hemorrhage. Liver microsomes prepared from TCDD-treated dams on day 20 of gestation exhibited a 2- to 3-fold increase in cytochrome P-450 content which was accompanied by a shift in the absorbance optimum of the dithionite reduced-CO spectrum to 448 nm. Catechol estrogen formation activity was decreased by 50–75% in hepatic microsomes from TCDD-treated dams. In contrast 7α-hydroxylation of testosterone increased nearly 4-fold, while 16α- and 6β-hydroxylase activities were unchanged in microsomes following exposure to TCDD. Thus, the inhibition of catechol estrogen formation associated with TCDD treatment did not reflect a general decrease in microsomal steroid hydroxylase activities. Insofar as catechol estrogen formation is physiologically a major pathway for estrogen metabolism, serum concentrations of 17β-estradiol were measured in a second group of pregnant rats treated with TCDD on days 4–15 of gestation. Serum estradiol levels were not different between control and treated dams at this stage of pregnancy. Thus, the present study does not support a link between TCDD-mediated inhibition of catechol estrogen formation measured *in vitro* in liver microsomes and altered circulating estradiol levels *in vivo* during pregnancy.

The environmental chemical 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a potent inducer of microsomal monooxygenase enzymes and associated drug metabolism [1, 2]. Steroid hormones are endogenous substrates for the liver monooxygenase system, and data from several laboratories indicate that TCDD administered in high doses significantly alters cytochrome P-450-dependent androgen metabolism in rat liver microsomes [3–5]. Based upon these data, Barsotti *et al.* [6] proposed that TCDD-mediated induction of estrogen and progesterone metabolism is the cause of altered hormonal balance and subsequent reproductive dysfunction. In contrast, we have observed that pretreatment of female rats with a low dose of TCDD was associated with significantly higher serum estrogen concentrations following exogenous administration of estrone [7]. Despite the observed increase in serum levels, however, the uterotrophic action of administered estrogen was not enhanced in TCDD-treated animals. Thus, these data indicate that short-term TCDD exposure was not associated with an alteration in estrogenic action in non-pregnant animals.

Studies of the *in utero* toxicity of TCDD have reported adverse effects on fetal development without significant maternal toxicity following oral administration of as little as 1–2 μg TCDD during pregnancy [8, 9]. There have been no reports, however, of the effect of TCDD on the oxidative metabolism of estrogens, nor studies to determine if steroid hydroxylase activities in liver microsomes are altered by exposure to these very low doses of TCDD during pregnancy. The initial objective of the present study was to evaluate the effect of administration of low, but fetotoxic doses of TCDD to pregnant rats on steroid metabolism in maternal liver microsomes. It was germane to further assess whether altered catechol estrogen formation activity *in vitro* correlated with serum estradiol levels *in vivo* following TCDD treatment.

MATERIALS AND METHODS

Animals. Pregnant Holtzman rats were housed in wire cages with food (Purina Rat Chow) and water *ad lib*. Animals were treated during pregnancy with TCDD, 1 $\mu g \cdot kg$ body $wt^{-1} \cdot day^{-1}$ by gavage, or with vehicle alone. TCDD was dissolved in corn oil–acetone (95:5). In experiment A, TCDD was administered on days 7–19 of gestation, and caesarian sections were performed on day 20 under pentobarbital anesthesia. In experiment B, animals were treated from days 4 to 15 of gestation and studied on days 16–17. The number of live and resorbed

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implants were recorded in each experiment, and all fetuses were examined for gross malformations.

Enzyme assays. Livers were excised and a 20% homogenate was prepared in 50 mM sodium phosphate (pH 7.4) buffer containing 1.15% KCl (w/v) with a Potter–Elvehjem homogenizer. The microsomal pellet was prepared from the 9,000 *g* supernatant fraction by centrifugation at 105,000 *g* for 60 min. Microsomes were resuspended in 0.1 M potassium phosphate buffer, pH 7.4, so that 1 ml contained microsomes from 1 g of liver.

Catechol estrogen formation was measured by a modification of the radioenzymatic method of Paul *et al.* [10]. Ethinylestradiol was used as the substrate because it is exclusively metabolized to cathechol products [11]. The incubation mixture contained 0.1 M potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 0.5 mM ascorbic acid, 0.5 mM ethinylestradiol substrate (in 0.002 ml ethanol), 0.5 mM NADPH, [^{3}H]*S*-adenosyl methionine (0.5 μCi/50 nmoles), 2 units catechol-*O*-methyl transferase (COMT, Sigma), and 0.03 to 0.06 μg microsomal protein in a final volume of 0.10 ml. All activities were corrected for values in the absence of NADPH. 2-Hydroxy [6,7-^{3}H]ethinylestradiol standard was synthesized by incubating [6,7-^{3}H]ethinylestradiol with mushroom tyrosinase [12]. The 2-hydroxy [^{3}H]ethinylestradiol product was then methylated with COMT and *S*-adenosyl methionine using the incubation system described above. The radiolabeled metabolite of ethinylestradiol formed by liver microsomes was characterized by TLC and found to comigrate as a single radioactive peak with the methylated 2-hydroxy [^{3}H]ethinylestradiol standard in the following systems: (A) cyclohexane–chloroform–acetic acid (40:50:10, by vol.) on silica gel plates, and (B) methanol–water (80:20) on C_{18} μBondapack reverse phase plates (Whatman).

Testosterone hydroxylase activity in liver microsomes was assayed in 0.1 M potassium phosphate buffer (pH 7.4) containing 10 mM $MgCl_2$, 1.0 mM NADPH, and 0.12 mM [^{14}C]testosterone (700,000 dpm added in 0.01 ml ethanol). Incubations were run for 10 min at 37° with 0.3 to 0.6 mg microsomal protein in a final volume of 1.0 ml. The radiolabeled products were extracted in methylene-chloride and separated by TLC on silica gel plates in chloroform–ethyl acetate–ethanol (4:1:0.7, by vol.) according to the method of Shiverick and Neims [13]. The radioactive zones were located by autoradiography, cut and counted in Aquasol. The radiochemical purity of the respective 16α-, 7α- and 6β-hydroxytestosterone metabolites was determined as previously described [13].

Cytochrome P-450 was measured by the dithionite reduced CO-difference spectrum according to Omura and Sato [14], and protein concentration by the biuret method [15]. Data were analyzed by Student's *t*-test.

Radioimmunoassay. Serum estradiol concentrations were determined by RIA [16] with a specific sheep antiserum provided by Dr. D. G. Niswender (Colorado State University). The sensitivity of this assay was 5 pg, and all samples were corrected for procedural losses. The inter-assay coefficient of variation (CV) was 15.5% and the intra-assay CV was 6.2%.

Safety. All laboratory personnel were instructed in the health hazards of TCDD and its safe handling. Prohibitions against eating, drinking and smoking in areas where TCDD was used were rigorously enforced. The entrance to rooms where TCDD was used was marked with biohazard signs designating TCDD as the hazard.

Materials. TCDD was supplied by Dr. L. Dale of the Environmental Protection Agency. [^{14}C]Testosterone (55 mCi/mmole) and [^{3}H]*S*-adenosyl methionine (68 Ci/mmole) were purchased from the New England Nuclear Corp. (Boston, MA). Ethinylestradiol, 16α-hydroxytestosterone and 6β-hydroxytestosterone were purchased from Steraloids (Wilton, NH); 7α-hydroxytestosterone was a gift from the British Medical Research Council. The following were purchased from the Sigma Chemical Co. (St. Louis, MO); *S*-adenosyl methionine, testosterone, ascorbic acid and catechol-*O*-methyl transferase. Silica gel 60F-254 plates (E. Merck, 0.25 mm), from Brinkmann Instruments (Westbury, NY), were prewashed in methanol and benzene. All reagents were nanograde quality from Mallinckrodt (St. Louis, MO).

RESULTS

Oral administration of 1 $\mu g \cdot kg^{-1} \cdot day^{-1}$ of TCDD to pregnant rats on days 7–19 of gestation significantly reduced maternal body weight gain during pregnancy from 143 ± 19 g ($\bar{X}$ ± S.E.) in control dams to 61 ± 15 g in treated animals ($P < 0.05$). Analysis of litters from TCDD-treated dams on day 20 showed malformations characterized by gastrointestinal hemorrhage, thin abdominal walls, and gross growth retardation. No visceral lesions were observed in control fetuses, while these abnormalities were noted in a total of 66% of fetuses in litters from four TCDD-treated dams. These data confirm previous reports of the fetotoxic effects of this dose of TCDD [8, 9].

Data in Table 1 show the composition of liver microsomes prepared from control and TCDD-treated dams on day 20 of gestation. Microsomes from TCDD-treated animals exhibited a 2- to 3-fold increase in cytochrome P-450 content accompanied by a shift in the wavelength optimum of the reduced CO-difference spectrum from 450 to 448 nm. In contrast, the protein content of microsomes was not different between groups.

Table 1. Effect of TCDD administration on days 7–19 of gestation on cytochrome P-450 and protein content of maternal liver microsomes*

	λ_{max} (nm)	Cytochrome P-450 (nmoles/g liver)	Protein (mg/g liver)
Control	450	4.9 ± 0.9	21.4 ± 0.1
TCDD	448	12.0 ± 1.4†	24.3 ± 2.9

* Animals were studied on day 20 of gestation. λ_{max} is the optimal wavelength of the reduced CO-difference spectrum of cytochrome P-450 in liver microsomes. Data are means ± S.E. of four animals in each group.

† $P < 0.01$.

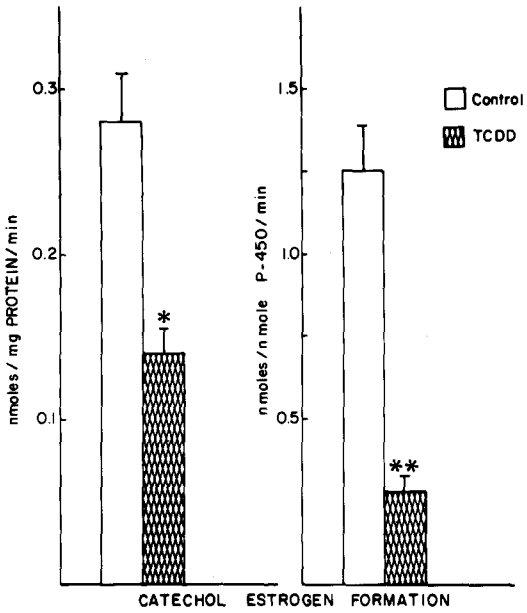

Fig. 1. Effects of TCDD administered on days 7–19 of gestation on catechol estrogen formation in liver microsomes from dams on day 20. Ring A hydroxylation of ethinylestradiol was assayed in 100 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 0.5 mM ascorbic acid, 0.5 mM ethinylestradiol substrate, 0.5 mM NADPH, [^{3}H]*S*-adenosyl methionine (0.5 μCi/50 nmoles), 2 units catechol-*O*-methyl transferase and 0.03 to 0.06 mg microsomal protein. Data are the means ± S.E. of four animals in each group. Key: (*) $P < 0.05$, and (**) $P < 0.001$.

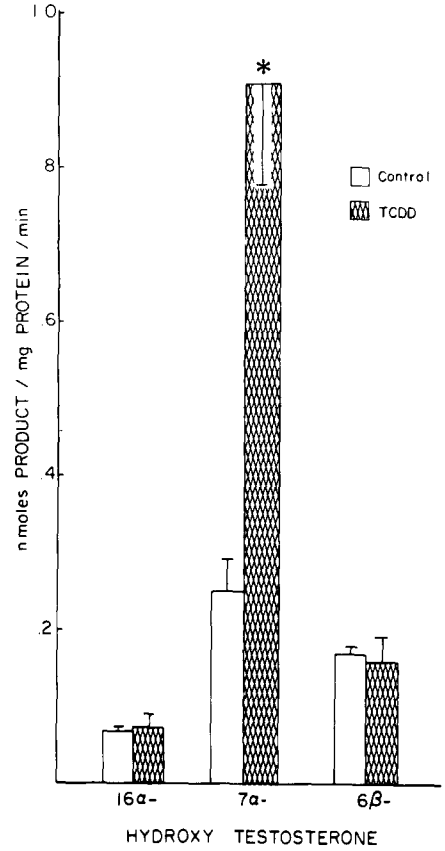

Fig. 2. Effects of TCDD administered on days 7–19 of gestation on liver microsomal testosterone hydroxylase activities. [^{14}C]Testosterone was metabolized to 16α-, 7α- or 6β-hydroxy testosterone products in 100 mM potassium phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 5 mM NADPH, 0.12 mM substrate and 0.3 to 0.6 mg microsomal protein. Data are the means ± S.E. of four animals in each group. Key: (*) $P < 0.001$.

Estrogen 2-hydroxylase activity in liver microsomes catalyzes the ring A hydroxylation of estrogen substrates to form a 2-hydroxy catechol estrogen product. Data in Fig. 1 show that catechol estrogen formation activity was decreased significantly in liver microsomes from TCDD-treated dams, by 50% when expressed per mg protein (specific activity), and by 75% when expressed per nmole P-450 (turnover number).

Several other pathways for the metabolism of steroids were also studied to determine whether the inhibitory effect of TCDD treatment on estrogen metabolism reflected a general decrease in microsomal steroid hydroxylase activities. Data in Fig. 2 show testosterone 16α-, 7α- and 6β-hydroxylase activities in liver microsomes from control and TCDD-treated dams. 7α-Hydroxylation of testosterone was increased nearly 4-fold in microsomes from treated animals, while hydroxylation of testosterone at the 16α- and 6β-positions was unaffected. Thus, TCDD administration is associated with a pattern of mixed induction and suppression of various steroid hydroxylase pathways.

Insofar as catechol estrogen formation is a major pathway for estrogen metabolism in the rat [11, 17], further experiments were conducted to determine whether circulating estrogen levels were altered by TCDD exposure. A second group of pregnant rats was treated with TCDD or vehicle from days 4 to 15 of gestation. Fetuses from TCDD-treated dams again showed a high incidence (41%) of visceral lesions characterized by gastrointestinal hemorrhage. Data in Fig. 3 show that serum concentrations of 17β-estradiol were not different between control and TCDD-treated dams. All values are in agreement with serum estradiol concentrations which have been reported by Taya and Greenwald [18] for days 16–17 of pregnancy.

DISCUSSION

The present study confirms previous reports on the significant fetotoxicity associated with oral administration of TCDD in 1 $\mu g \cdot kg^{-1} \cdot day^{-1}$ quantities during gestation in rats [8, 9]. The biochemical mechanism of TCDD-mediated reproductive dysfunction and fetotoxicity is presently unknown. In view of the well known actions of TCDD to induce liver drug metabolism [2, 19], it has been suggested that TCDD interferes with endocrine function by altered metabolism of steroid hormones [6]. Data from several laboratories indicate that the administration of 80 μg/kg doses of TCDD was associated with inhibition of certain androgen hydroxylase activities in liver microsomes from male rats [3–5],

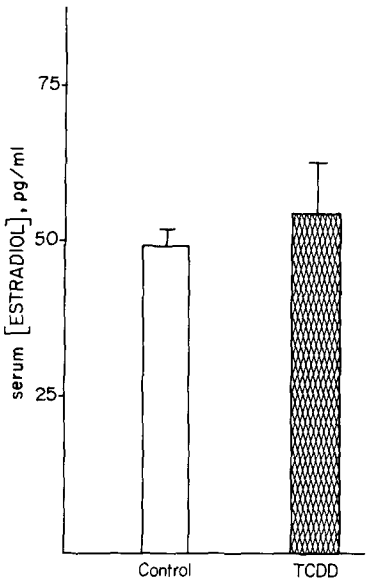

Fig. 3. Serum concentrations of 17β-estradiol in pregnant rats following administration of TCDD on days 4–15 of gestation. Data are the means ± S.E. of four animals in each group.

while these enzyme activities were increased 3- to 5-fold in microsomes from female rats [4]. In contrast, we have reported that the short-term administration of very low doses of TCDD significantly impaired the *in vivo* disposition and/or metabolism of exogenous estrone [7]. The effects of this dose of TCDD on the oxidative metabolism of steroid hormones in liver microsomes has not been studied previously.

The objective of the present study was to evaluate the effects of exposure to very low, but fetotoxic doses of TCDD on cytochrome P-450-dependent steroid hydroxylation in hepatic microsomes from pregnant rats. The quantity of TCDD administered in the present study has been associated with a 20-fold increase in aryl hydrocarbon hydroxylase (AHH) activity in liver microsomes from non-pregnant rats [7]. The TCDD-mediated increase in cytochrome P-450 content of liver microsomes was found in the present study to be accompanied by a shift to a 448 nm spectral optimum, data which confirm previous reports that TCDD is a potent inducer in pregnant rats [20]. Catechol estrogen formation activity in liver microsomes was decreased significantly by this dose of TCDD, while testosterone 7α-hydroxylation was greatly enhanced without any change in 16α- and 6β-testosterone hydroxylase activities. Such mixed induction–suppression effects of TCDD have been reported by others for a number of pathways of hepatic drug matabolism [1, 21] and appear to reflect the multiple forms of microsomal monooxygenase enzymes and their selective regulation.

The second question under investigation was whether estrogen metabolism *in vitro* in liver microsomes is related to serum estradiol concentrations *in vivo* following TCDD administration. Ring A hydroxylation of estrogens to catechol products is the main pathway for the oxidative metabolism of estrogens in the rat [11, 17]. If TCDD exposure has any physiological effect on steroid hormone balance, the *in vitro* data suggest that it would be in the direction of impaired estrogen metabolism. These data support our earlier report [7] that TCDD exposure impaired the *in vivo* disposition and/or metabolism of a pharmacologic dose of estrone in non-pregnant animals. In the present study, however, serum concentrations of 17β-estradiol were not altered in TCDD-treated dams, data which suggest that impaired microsomal metabolism *in vitro* may not be related to an alteration in endogenous levels of circulating estrogen *in vivo* during pregnancy. Alternatively, TCDD may selectively induce other pathways of estrogen metabolism in pregnant animals, as has been reported recently by Brock and Vore [22] for hepatic estrone glucuronyl transferase activity. An additional possibility is that serum estradiol concentrations in TCDD-treated dams may remain unchanged due to homeostatic regulation of endogenous estrogen biosynthesis in response to altered hepatic metabolism.

In summary, biochemical mechanisms other than liver steroid metabolism may be responsible for the reproductive toxicity of TCDD. In view of earlier work which dissociates serum estrone levels from uterotrophic action in TCDD-treated non-pregnant animals [7], the possibility exists that circulating estradiol concentrations may not be a valid indicator of estrogenic action in pregnant rats following TCDD exposure. Thus, levels of plasma binding proteins, possibly α-fetoprotein in pregnant animals, may be more important regulators of estrogenic action [23]. Alternatively, TCDD has been reported to have direct effects on gonadal tissue, as well as to induce aryl hydrocarbon hydroxylase activity in the ovary, placenta, and fetal tissues [20, 24, 25]. Thus, the mechanism of TCDD-associated reproductive dysfunction and fetotoxicity may involve multiple variables.

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