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Effect of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin Administration and High-Fat Diet on the Body Weight and Hepatic Estrogen Metabolism in Female C3H/HeN Mice¹

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Abstract

We studied the effect of administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) by i.p. injection once every two weeks in combination with a high-fat (HF) diet for 8 or 16 weeks on the body and organ weight changes as well as on the hepatic enzyme activity for estrogen metabolism in C3H/HeN female mice. Administration of TCDD at 100 µg/kg b.w. once every two weeks for 8 weeks increased the body weight by 46% in the HF diet-fed animals, but not in the regular diet-fed animals. This is the first observation suggesting that TCDD, a potent Ah receptor agonist, may have an obesity-inducing effect in animals fed a HF diet. While TCDD increased liver weight and decreased thymus weight in animals, these effects were enhanced by feeding animals a HF diet. Metabolism studies showed that TCDD administration for 8 or 16 weeks increased the liver microsomal activity for the 2- and 4-hydroxylation of 17β-estradiol in animals fed a control diet, but surprisingly not in animals fed a HF diet. Treatment with TCDD dose-dependently increased the hepatic activity for the *O*-methylation of catechol estrogens in both control and HF diet-fed animals, and it also decreased the levels of liver microsomal sulfatase activity for hydrolysis of estrone-3-sulfate. TCDD did not significantly affect the hepatic enzyme activity for the glucuronidation or esterification of endogenous estrogens. It is suggested that enhanced metabolic inactivation of

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endogenous estrogens by hepatic estrogen-metabolizing enzymes in TCDD-treated, control diet-fed animals contributes importantly to the reduced incidence of estrogen-associated tumors in animals treated with TCDD.

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)⁶, an accidental byproduct formed during the manufacturing and incineration process of chlorine-based products, is generally considered to be among the most potent environmental toxicants known to date. Contamination of TCDD in the environment is widespread, and its resistance to biodegradation results in its presence in the environment [1] and in human body for long periods of time [2]. TCDD is a developmental and reproductive toxicant and causes immunotoxicity, dermal and hepatic toxicity, and a plethora of endocrine-disrupting effects [3–8]. In addition, TCDD is also considered a carcinogen, increasing the incidence of a variety of cancers in humans as well as in laboratory animals [4,9–13]. Tumors of the breast are common in humans, but at present epidemiological data are conflicting with respect to the link between TCDD exposure and the risk of breast cancer. In comparison, the data from earlier animal studies were clearer and more intriguing. While chronic administration of TCDD to female rats enhanced the tumor incidence in liver and also many other organ sites, this treatment inhibited the formation of spontaneous tumors in the uterus, mammary gland, and pituitary [11–13], which are target tissues for endogenous estrogens.

The mechanism for the unexpected inhibitory effect of TCDD on the development of tumors in various estrogen target organs in rodents is still not quite understood at present. A number of possible mechanisms, nevertheless, have been suggested for the anti-estrogenic actions of TCDD, which include stimulation of estrogen metabolism [14,15], a possible direct antiestrogenic action [13,16,17], and also possible alterations of the binding interaction between the estrogen receptor and its response element [18,19]. Since TCDD is an extremely potent agonist for the aryl hydrocarbon receptor (AhR), which mediates the induction of a number of drug-metabolizing enzymes in liver as well as in extrahepatic tissues [20], it has been widely considered that chronic TCDD administration may alter the multiple pathways of endogenous estrogen metabolism in certain ways that may contribute to an altered hormonal activity and also tumor incidence in various estrogen target organs.

Metabolically, it is known that the endogenous estrogens, such as 17 β -estradiol (E₂) and estrone (E₁), can undergo extensive metabolism *in vivo*, including oxidation (mostly catalyzed by cytochrome P450 enzymes) and conjugation (such as glucuronidation, sulfonation, and *O*-methylation) [21,22]. There is a considerable body of experimental evidence suggesting that some of the endogenous estrogen metabolites, such as 4-OH-E₂ and 2-methoxy-E₂ (an *O*-methylated product of 2-OH-E₂ formed by catechol-*O*-methyltransferase), may play important roles in the causation and/or prevention of estrogen-induced cancers [21,23–25]. Some of the earlier studies have shown that a beneficial modulation of the endogenous estrogen metabolism is correlated with a reduced risk for estrogen-induced cancer in animal models and also humans. For instance, studies by Bradlow and his colleagues showed that chronic administration of indole-3-carbinol stimulated the 2-hydroxylation of E₂ [26–29] and inhibited the development of mammary preneoplasia and spontaneous mammary tumors in female C3H/OuJ mice [30, 31]. A similar inhibitory effect of indole-3-carbinol on the formation of spontaneous endometrial cancer was also observed in female rats [30]. Recent studies showed that chronic administration of sodium phenobarbital (a prototypic inducer of hepatic drug/steroid-

⁶Abbreviations used: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; E₂, 17 β -estradiol; E₁, estrone; 2-OH-E₂ and 4-OH-E₂, 2- and 4-hydroxyestradiol, respectively; COMT, catechol-*O*-methyltransferase; HF, high-fat diet; AhR, aryl hydrocarbon receptor.

metabolizing enzymes) significantly inhibited the formation of E₂-induced mammary tumors in female ACI rats [33]. Metabolic analysis showed that this tumor-inhibitory effect of sodium phenobarbital was accompanied by a several-fold increase in hepatic E₂ 2-hydroxylation but with little or no change in its 4- and 16 α -hydroxylation, while the plasma levels of E₂ were unchanged in these animals [33].

Therefore, we sought to determine in the present study the effect of administration of TCDD in combination with a high-fat (HF) diet for 8 or 16 weeks on the body and organ weights as well as on the hepatic metabolizing enzyme activities catalyzing the multiple pathways of estrogen metabolism in the C3H/HeN female mice. These experiments were, in part, aimed to provide evidence for the hypothesis that beneficial alterations of the multiple metabolic pathways of endogenous estrogens are a significant factor contributing to the observed protective effect of TCDD against various estrogen-associated tumors in rodents [11–13]. It is of note that the caloric contribution of the fat component in the high-fat diet used in this study was 39% as opposed to 12% in the control diet. There were a number of reasons for the inclusion of a HF diet as a subgroup in the present study: (i) Studies have demonstrated that certain fatty acids and/or their metabolites can serve effectively as natural ligands for some of the nuclear receptors which regulate the expression of drug-metabolizing enzymes as well as liver growth [34–36]. (ii) Recent epidemiological studies have suggested that obesity is an important risk factor for human breast cancer [37,38], but little is known about the modulating effect of a HF diet on hepatic estrogen metabolism as well as on the metabolizing enzyme-inducing activity of AhR ligands. Therefore, we believed it would be of interest to also determine the potential interactions between dietary fatty acids and TCDD with respect to their effects on animal body and organ weights as well as on hepatic enzyme levels for estrogen metabolism.

Materials And Methods

Chemicals

17 β -Estradiol (E₂), estrone (E₁), NADPH, and UDPGA triammonium salt were obtained from the Sigma Chemical Co. (St. Louis, MO). 2-OH-E₂ and 4-OH-E₂ were obtained from Steraloids, Inc. (Newport, RI). TCDD (>99.5% pure) was obtained from Cambridge Isotope Laboratory (Andover, MA). [6,7-³H]E₂ and [6,7-³H]E₁ (specific activities of 51 and 58 Ci/mmol, respectively) and [4-¹⁴C]E₂ (specific activities of 55.1 mCi/mmol) were purchased from PerkinElmer Life Science Products (Boston, MA). All the organic solvents used in this study were of HPLC grade or better, and were obtained from Fisher Scientific (Atlanta, GA).

All the glass test tubes used in the present study were silanized with 5% (v/v) dimethyldichlorosilane in toluene for 10 min followed by rinses in pure toluene twice and pure methanol three times. The test tubes were allowed to dry at room temperatures and then thoroughly rinsed with distilled water.

The control AIN-76A diet (D10011) and the high-fat (HF) diet (D12370, also called the Newmark Stress Diet B) were prepared by Research Diets (New Brunswick, NJ). Their compositions were given in Table 1. The caloric contribution of the protein, carbohydrate and fat component in the control AIN-76A diet (3.9 kcal/gram diet) was 21%, 68%, and 12%, respectively, and their respective contribution in the HF diet (4.6 kcal/gram diet) was 21%, 40%, and 39%.

Treatment of animals with TCDD

The mammary tumor virus (MTV)-positive C3H/HeN female mice (at 3 weeks of age) were purchased from Charles River (Boston, MA). Upon arrival, the animals were allowed to acclimatize to the new environment for two weeks. Then the animals were randomly divided

into two large groups, with one receiving the control diet and the other receiving the HF diet. In each of the diet group, the animals were subdivided into several small groups (5 to 6 animals per group) and they were treated i.p. with different doses of TCDD once every two weeks for 8 or 16 weeks. The TCDD was dissolved in corn oil at 0.18, 1.8 and 18 $\mu\text{g/mL}$ (by heating the solution to 50°C followed by brief sonication), and the animals were injected i.p. with these TCDD solutions (at 5.56 μL for every gram of body weight), which gave the final dosage of 1, 10 and 100 $\mu\text{g/kg}$ b.w. The control animals were injected the same volume of vehicle (corn oil) alone. The doses of TCDD were selected for testing in the present study were largely based on earlier studies showing that this broad dose range of TCDD was suitable for studying the toxicity and enzyme induction patterns in most sensitive strains of mice [39]. Note that the high dose level (100 $\mu\text{g/kg}$ b.w., once every two weeks) was only used in the 8-week experiment given the possibility that TCDD at this high dose might be significantly more toxic when it was given to animals for 16 weeks. Throughout the experiment period, the animals had free access to food and water. Animals were killed by euthanasia with CO_2 overdose, and their organs (liver, uterus, thymus, and brain) were immediately removed for determination of wet weights and then snap-frozen in liquid nitrogen. The tissue samples were stored at -80°C until use. The study described here that involved the use of live animals was approved by the IACUC of Rutgers University and UMDNJ (Piscataway, NJ). The animal experiment was conducted once in the present study.

Preparation of liver microsomes and cytosols

Liver tissues collected from the control group or from each of the treatment groups (5–6 animals per group) were randomly pooled into two subgroups with 2–3 liver samples in each subgroup. Liver microsomes and cytosols were prepared by differential centrifugations as previously described [40]. Aliquots (0.5 mL) of microsomal suspensions in 0.25 M sucrose were stored at -80°C . The protein content was determined using the BioRad protein assay kit with bovine serum albumin (BSA) as standard.

Assay of the NADPH-dependent 2- and 4-hydroxylation of E_2 by liver microsomes

The reaction mixture consisted of liver microsomal protein (1 mg/mL), $[4-^{14}\text{C}]\text{E}_2$ (dissolved in 2 μL ethanol), 2 mM NADPH and 5 mM ascorbic acid in a final volume of 1.0 mL Tris-HCl (100 mM)/HEPES (50 mM) buffer, pH 7.4. The reaction was initiated by addition of microsomal protein and lasted for 30 min at 37°C with constant mild shaking. The reaction was arrested by placing tubes on ice followed immediately by two extractions with 7 mL of ice-cold ethyl acetate. The overall extraction rate for the total radioactivity contained in each tube was usually between 85–95%. Note that when several representative non-radiolabeled estrogens, including E_2 , E_1 , 2-OH- E_2 and 2-OH- E_1 , were tested individually, each of them was found to have a similar extraction rate (91–96%). The combined organic supernatants were dried under a stream of nitrogen. The resulting residue was re-dissolved in 100 μL of 50% aqueous methanol, and a 50 μL aliquot was analyzed for metabolite composition by HPLC.

The HPLC analysis of E_2 and E_1 metabolites was performed as described previously [40]. The solvent system for separation of E_2 and its metabolites consisted of acetonitrile (*solvent A*), 0.1% acetic acid in water (*solvent B*), and 0.1% acetic acid in methanol (*solvent C*). The modified solvent gradient (*solvent A/solvent B/solvent C*) used for eluting estrogen metabolites from the column was as follows: 8 min of isocratic at 16/68/16; 7 min of concave gradient (curve number 9) to 18/64/18, 13 min of concave gradient (curve number 8) to 20/59/21, 10 min of convex gradient (curve number 2) to 22/57/21, 13 min of concave gradient (curve number 8) to 58/21/21, then followed by a 0.1-min step to 92/5/3 and 5 min of isocratic at 92/5/3. The gradient was then returned to the initial condition (16/68/16) and held for 15 min before analysis of the next sample. HPLC metabolite profiles were obtained by radioactivity measurements.

Assay of the UDPGA-dependent glucuronidation of E₂ by liver microsomes

The conditions for microsomal glucuronidation of estrogens were the same as described previously [41]. The reaction was initiated by addition of microsomal protein and lasted for 15 min at 37°C. The reaction was arrested by placing the test tubes on ice and adding 0.5 mL ice-cold 50 mM Tris-HCl solution (pH 8.75) followed immediately by two extractions with 7 mL toluene to separate unconjugated estrogen from water-soluble estrogen glucuronides. The toluene-phase, which contained the unconjugated estrogen, was removed. Portions (200 µL) of the aqueous phase, which contained glucuronidated estrogen metabolites, were carefully removed and assayed for radioactivity content in a Beckman liquid scintillation spectrometer. The blank values, obtained from incubations in the absence of UDPGA, were determined in each individual assay and subtracted.

Assay of the enzymatic desulfation of E₁-3-sulfate

E₁-3-sulfatase activity in hepatic microsomes of female C3H/HeN mice was determined by measuring the formation of radioactive E₁ from [6,7-³H]E₁-3-sulfate (the substrate) as described previously [42,43]. The incubation mixture consisted of rat liver microsomal protein (0.1 to 0.5 mg/mL) and 50 µM E₁-3-sulfate (containing 0.2–1.0 µCi of [6,7-³H]E₁-3-sulfate) in a final volume of 0.5 to 1.0 mL Tris-HCl buffer (25 mM, pH 7.4). The incubation was carried out at 30°C for 10 to 15 min. The reaction was arrested by immediately placing the incubation tubes on ice followed by extraction with 5 mL ice-cold toluene. Aliquots of the organic supernatants were removed for radioactivity measurement. Blank values were determined for each individual assay in the absence of subcellular proteins and were subtracted.

Assay of the COMT-mediated O-methylation of 2-OH-E₂ and 4-OH-E₂

The reaction mixture was prepared at ~4°C and consisted of a 30 µM concentration of 2-OH-E₂ or 4-OH-E₂, 1.0 mg/mL mouse liver cytosolic protein, 50 µM *S*-adenosyl-*L*-methionine (containing 0.25 µCi of *S*-[methyl-³H]adenosyl-*L*-methionine), 1.2 mM MgCl₂, and 1.0 mM dithiothreitol in a final volume of 1 mL of Tris-HCl buffer (10 mM, pH 7.4) [44,45]. The reaction was initiated by addition of liver cytosolic protein and was carried out at 37°C for 20 min under constant mild shaking. The reaction was arrested by placing the tubes on ice followed immediately by addition of 0.5 mL of ice-cold water and extraction of the methylated catechol estrogens with 5 mL ethyl acetate. After centrifugation for 20 min at 1000 g, portions of the organic supernatants (2 mL) were accurately removed for measurement of radioactivity in a scintillation counter (model LS 5000TD; Beckman Instruments, Berkeley, CA). Blank values obtained from incubations without cytosolic protein were determined in each individual assay and subtracted. The blank radioactivity counts were usually <1/10 of the values obtained from incubations in the presence of cytosolic protein.

Assay of the enzymatic formation of E₂-17β-oleate

The reaction mixtures contained 50 µM [³H]E₂ (1–5 µCi), 100 µM oleoyl-CoA, 5 mM magnesium chloride in 0.1 M sodium acetate buffer (pH 4.0–8.0) in a glass test tube. For the preparation of the incubation mixture, radioactive E₂ in ethanol was added first, dried under nitrogen, and then the remaining components of the incubation mixture (including nonradioactive E₂ in 5 µL of ethanol) were added. This procedure resulted in uniform distribution of radioactive and nonradioactive E₂ throughout the incubation mixture. In some experiments, 1 nM [³H]E₂ was used for the incubations. The reaction was initiated by addition of hepatic microsomes (1 mg/mL of liver microsomal protein). The final volume of the incubation mixture was 0.5 mL. After incubation at 37°C for 30 min, the reaction was arrested by placing the tubes on ice, followed by addition of 0.5 mL of ice-cold sodium acetate buffer (pH 5.5) and 5 mL of ethyl acetate (HPLC grade from Fisher Scientific). The samples were vortexed immediately and centrifuged for 10 min at 3000g. The organic phase was removed,

and the extraction was repeated a second time. The organic solvent extracts were combined and evaporated to dryness under a stream of nitrogen. Each resulting residue was dissolved in 100 μ L of methanol and analyzed by HPLC.

The measurement of [3 H]E₂-17 β -oleate was done by using HPLC on a Spherisorb ODS column (5- μ m particle size, 250 $\times$ 4.6 mm i.d.). The HPLC system consisted of a Waters (Milford, MA) 600E solvent gradient programmer, a Waters Lambda-Max model 481 UV detector (set at 280 nm), and a radioactive flow detector (β -RAM from the IN/US, Fairfield, NJ) with a liquid cell. The solvent system consisted of acetonitrile/H₂O with 0.1% acetic acid/methanol. The solvent gradient used for elution of the compounds from the column was as follows: 12-min isocratic at 30/6/64; 6-min with a 10 convex gradient to 60/0/40; 15-min isocratic at 60/0/40; 2-min with a 2 convex gradient to 20/0/80; 5-min isocratic at 20/0/80, and the column was then returned to initial conditions over 15 min. The flow rate was 1.2 mL/min. The retention times of the radioactive metabolites agreed exactly with corresponding UV-absorbing peaks. Metabolite quantification was based on the amount of radioactivity in the metabolite peak as compared to the total radioactivity collected from the HPLC column from each sample.

Statistical analysis

All data were presented as mean $\pm$ S.D. For the body and organ weight data, Tukey's test was performed to determine the difference between the control group and the TCDD-treated group. However, for the enzyme activity data, one-way analysis of variance followed by Student's *t* test was used to determine the difference between the control group and the TCDD-treated group. A *P* value of <0.05 was considered statistically significant.

Results

Effect of TCDD on animal body and organ weight (Fig. 1)

Control diet groups—Administration of TCDD at 1, 10, or 100 μ g/kg b.w. to control diet-fed animals once every two weeks for 8 weeks or at 1 or 10 μ g/kg b.w. for 16 weeks had no appreciable effect on the body weight (Fig. 1A, 1B). After 8 weeks of TCDD treatment, the liver weight was increased in a dose-dependent manner, with a maximum of 51% increase over the control animals at the highest TCDD dose tested (100 μ g/kg b.w.) (Fig. 1E). In comparison, when TCDD treatment (at 1 and 10 μ g/kg b.w. only) was extended to 16 weeks, the liver weight increase appeared to be not dose-dependent (Fig. 1F). The thymus weight in these control diet-fed animals was decreased by TCDD treatment for 8 weeks, in a dose-dependent manner, and the maximal reduction of thymus weight was 62% after 8-week treatment with 100 μ g/kg b.w. TCDD (Fig. 1I). The thymus weight was also reduced after 16 weeks of treatment with TCDD (10 μ g/kg b.w.) (Fig. 1J). The uterine weight in most of the animals that were fed a control diet and treated with different doses of TCDD for 8 or 16 weeks was not significantly different from the control untreated animals, except that there was a 40% increase (*P* < 0.05) in animals treated with 10 μ g/kg b.w. TCDD for 16 weeks (Fig. 1M, 1N).

HF diet groups—Administration of TCDD at 1 or 10 μ g/kg b.w. to HF diet-fed animals once every two weeks for 8 or 16 weeks did not significantly affect the animal body weight, but surprisingly, TCDD at a higher dose (100 μ g/kg b.w.) increased the body weight in HF diet-fed animals by 46% over the control animals (*P* < 0.05; Fig. 1C). The liver weight in the HF diet-fed animals treated with TCDD for 8 or 16 weeks was increased; the effect was stronger in the HF diet-fed animals than in the control diet-fed animals (Fig. 1G, 1H). The thymus weight in the HF diet-fed animals was decreased by treatment with TCDD for 8 weeks in a dose-dependent manner (Fig. 1K), but its effect in animals fed a HF diet for 16 weeks was reduced (Fig. 1L). The uterine wet weight in most TCDD-treated groups was not significantly

different from the corresponding control animals, but there was a 40% decrease in animals (HF group) treated with 100 µg/kg b.w. TCDD for 8 weeks ($P < 0.05$).

Effect of TCDD on hepatic estrogen-metabolizing enzymes

2- and 4-Hydroxylation of E₂ (Fig. 2)—Treatment of control diet-fed animals with TCDD at 1, 10, or 100 µg/kg b.w. for 8 weeks or at 1 or 10 µg/kg b.w. for 16 weeks increased liver microsomal activity for both 2- and 4-hydroxylation of E₂. The increase was significant when the animals were treated with a low dose of TCDD (1 µg/kg b.w.) for either 8 or 16 weeks. The maximal induction was approximately 80% over the control values when the animals were treated with 10 µg/kg b.w. TCDD for 8 weeks. However, when the animals were treated with 100 µg/kg b.w. of TCDD, the increase in the hepatic E₂ 2- and 4-hydroxylase activity was significantly less than what was seen with the 10 µg/kg b.w. dose.

Interestingly, when the animals were fed a HF diet for 8 or 16 weeks, treatment with 1 or 10 µg/kg b.w. of TCDD only produced a very modest increase in the levels of liver microsomal enzyme activity for the 2- or 4-hydroxylation of E₂; administration of TCDD at 100 µg/kg b.w. for 8 weeks actually decreased liver microsomal activity for E₂ oxidative metabolism ($P < 0.05$ for the reduction in E₂ 2-hydroxylation).

O-Methylation of catechol estrogens (Fig. 3)—Treatment of control diet-fed animals with TCDD markedly increased the hepatic cytosolic activity of catechol-*O*-methyltransferase (COMT) for the *O*-methylation of 2-OH-E₂ and 4-OH-E₂ in a dose-dependent manner. The maximal increase was approximately 100% over the control when the animals were treated with 100 µg/kg b.w. of TCDD for 8 weeks. The stimulatory effect after 16 weeks of TCDD treatment appeared to be less pronounced compared to what was seen with the 8-week treatment groups. The inducing effect of TCDD treatment on the hepatic COMT activity for the *O*-methylation of 2-OH-E₂ and 4-OH-E₂ was not affected by feeding animals a HF diet for 8 or 16 weeks.

Formation of E₂-17β-oleate (Fig. 4)—In control diet-fed animals, treatment with TCDD increased the liver microsomal activity for the formation of E₂-17β-oleate (a representative endogenous E₂-17β-fatty acid ester) by 10–20%. Treatment of HF diet-fed animals with TCDD for 8 or 16 weeks also had a similarly small effect on the hepatic enzyme activity for the formation of the E₂-17β-oleate.

UDPGA-dependent glucuronidation of E₂ and E₁ (Fig. 5)—Treatment of control diet-fed animals with TCDD only weakly increased the liver microsomal activity for the UDPGA-dependent glucuronidation of E₂ and E₁ (5–25%). Treatment of HF diet-fed animals with TCDD for 8 or 16 weeks also had a similarly weak inducing effect on liver microsomal glucuronidation of E₂ and E₁.

Desulfation of E₁-3-sulfate (Fig. 6)—Treatment of control diet-fed animals with TCDD at 1, 10, or 100 µg/kg b.w. for 8 weeks or at 1 or 10 µg/kg b.w. for 16 weeks decreased liver microsomal sulfatase activity for hydrolysis of E₁-3-sulfate in a dose-dependent manner. The decrease was very significant even when the animals were treated with the lowest dose of TCDD used (1 µg/kg b.w.) for either 8 or 16 weeks. The maximal reduction was approximately 40% when the animals were treated with 10 or 100 µg/kg b.w. of TCDD for 8 weeks. The effect of TCDD administration (at 1 or 10 µg/kg b.w.) in HF diet-fed animals was similar to what was observed in the control diet-fed animals.

Effect of HF diet on animal body and organ weight and on estrogen metabolism

For convenience, the effect of feeding animals a HF diet alone (without TCDD administration) for 8 or 16 weeks on animal body and organ weights and also on various hepatic estrogen-metabolizing enzymes was separately summarized below (Fig. 7).

Compared to animals fed a control diet, the body weight of animals fed a HF diet for 8 or 16 weeks was not statistically significant (Fig. 7A). Similarly, the wet weights of the liver, thymus and uterus from animals fed a HF diet for 8 or 16 weeks were also not significantly different from animals fed a control diet (Fig. 7B, 7C, 7D). Animals fed a HF diet alone for 8 or 16 weeks had approximately 30% and 37%, respectively, higher hepatic microsomal activity for the 2- and 4-hydroxylation of E_2 compared to the control diet-fed animals ($P < 0.05$) (Fig. 7G, 7H). However, animals fed with a HF diet for 8 or 16 weeks had a 25–35% decrease in the hepatic cytosolic COMT activity for the O-methylation of 2-OH- E_2 and 4-OH- E_2 ($P < 0.05$) (Fig. 7E, 7F).

Feeding animals a HF diet for 8 or 16 weeks decreased the hepatic microsomal activity for the formation of E_2 -17 β -oleate by 10–15% ($P < 0.05$) (Fig. 7I). In comparison, treatment with the HF diet did not appreciably affect the hepatic microsomal enzyme activity for the glucuronidation of E_2 and E_1 (Fig. 7J, 7K). The estrogen sulfatase activity was only decreased by approximately 10% by feeding the animals with a HF diet for 8 weeks, but the enzyme activity was not different after feeding the HF diet for 16 weeks (Fig. 7L).

Discussion

Effect of TCDD and HF diet on animal body and organ weight

Body weight—It has been well-documented that TCDD can cause a dose-dependent loss of body weight, or wasting syndrome, which is a characteristic phenomenon often observed in animals exposed to TCDD [46–49]. The weight loss usually manifests itself within a few days after exposure, and results in a substantial reduction of the adipose and muscle tissue [46,50]. Earlier studies have suggested that the body weight loss played a greater role in causing death in Sprague-Dawley rats and guinea pigs than in Fischer 344 rats and C57Bl/6 mice.

Interestingly, the data of our present study showed that in animals fed a control diet, treatment with TCDD at 1, 10, or 100 $\mu\text{g/kg}$ b.w. for 8 weeks or at 1 or 10 $\mu\text{g/kg}$ b.w. for 16 weeks did not reduce the animal body weight. Surprisingly, administration of TCDD at 100 $\mu\text{g/kg}$ b.w. increased the body weight in animals fed a HF diet by 46% ($P < 0.05$), but this effect was not observed in animals fed a normal diet. This finding is the first observation suggesting that TCDD, a potent AhR agonist, may have an obesity-inducing effect in the female C3H/HeN mice fed a HF diet.

Here it is of note that the effect of TCDD on the body weight in animals fed a HF diet for 8 weeks did not appear to be dose-dependent (see Fig. 1C, 1G). Although the possibility could not be completely ruled out that the lack of an effect of TCDD at the 10 $\mu\text{g/kg}$ b.w. dose might be due to experimental variations, it was very unlikely that this was due to errors involving TCDD administration because other parameters obtained from animals of the same treatment group, such as the TCDD-induced thymus weight decrease (Fig. 1K) and changes in the hepatic COMT activity (Fig. 3) or estrogen sulfatase activity (Fig. 6), were all dose-dependent, which suggest the presence of increasing actions of TCDD in these animals.

Mechanistically, it is speculated that animals fed a HF diet might have increased levels of certain fatty acids or their derivatives in liver as well as other organs. Since some of the fatty acids and/or their metabolites can serve effectively as endogenous ligands for certain nuclear receptors, such as the peroxisome proliferator-activated receptors (PPARs) [34–36], it is thus

suggested that the activation of certain PPAR signaling pathways by fatty acids may be involved in alterations of lipid metabolism and subsequently body weight change. In line with this possibility, recent studies have shown that there are cross-talks between the AhR system and the PPAR receptor system as well as with other nuclear receptor systems (discussed in ref. [51]).

Here it is also of relevance to note that many of the Vietnam War veterans were exposed to high levels of TCDD [52,53], and significant residues of this chemical have been found in their serum samples even after many years [38]. One of the most consistently identified diseases that were associated with TCDD exposure is the Type 2 diabetes mellitus [54–56]. Similar observations were also made in epidemiologic studies of other human subjects that were exposed to higher levels of TCDD [57–60]. However, the mechanism by which TCDD causes human Type 2 diabetes is not understood. It was noted in some of the epidemiological studies that there lacked a dose-response relationship between the TCDD levels in exposed population and the incidence of diabetes. The results of our present study showed that while TCDD had an obesity-inducing effect in animals fed a HF diet, it did not have an appreciable effect in animals fed a regular diet. This observation is of interest, and it may suggest that the ability of TCDD to induce obesity and perhaps other changes associated with the development of metabolic syndrome or diabetes may require the cooperation of other factors (such as high levels of certain fatty acids and/or their metabolites which may be needed for the activation of PPAR systems). It is not known at present as to how AhR activation by TCDD may lead to altered lipid metabolism and accumulation in animals fed a HF diet. More studies are needed to determine which energy metabolism pathways are affected by the AhR signaling system and also how fatty acids may interact with the AhR signaling system.

Lastly, it is of note that the results of our present study suggest that the parameter of body weight decrease, which is widely-used as a reliable gross parameter for chemical-induced toxicity in most animal models, may not serve as a good indicator for the levels of TCDD toxicity under certain conditions and in certain strains of mice.

Organ weight changes—In agreement with earlier studies [3,4], our data also showed that liver weight in the animals treated with TCDD for 8 or 16 weeks was increased. Notably, the effect of TCDD in inducing hepatomegaly was stronger in the HF diet groups than in the control diet groups. Thymic atrophy has also been found in nearly all animal species treated with lethal doses of TCDD [3,4]. As expected, the thymus weight was also found in the present study to be markedly decreased by TCDD treatment for 8 or 16 weeks. Compared with the animals fed a control diet, the effect of TCDD on thymus weight change was also more pronounced in animals fed a HF diet.

The data of our present study did not show a clear uniform change in uterine wet weight in animals treated with TCDD, but it was noted that there was a 40% decrease in animals (HF group) treated with 100 µg/kg b.w. TCDD for 8 weeks ($P < 0.05$) and there was a 40% increase in animals (control diet group) treated with 10 µg/kg b.w. TCDD for 16 weeks ($P < 0.05$). Mechanistically, a reduction of the uterine wet weight in animals treated with a high dose of TCDD (100 µg/kg b.w.) might be due to increased metabolic disposition of the endogenous estrogens *in vivo*, but the increase in the uterine wet weight seen in animals treated with a lower dose of TCDD (10 µg/kg b.w.) for 16 weeks is not quite understood.

Effect of TCDD and HF diet on hepatic estrogen metabolism

In the present study, we determined the effect of administration of TCDD on the hepatic enzymes involved in the multiple pathways of estrogen metabolism in C3H/HeN female mice that were fed either a regular diet or a HF diet. Our data showed that treatment of control diet-fed animals with TCDD (at 1 and 10 µg/kg b.w.) for 8 or 16 weeks increased liver microsomal

activity for both 2- and 4-hydroxylation of E₂, with a maximal induction of approximately 80% over the control values. The overall extent of induction of hepatic enzyme activity for E₂ hydroxylation was moderate compared to other chemical inducers (such as phenobarbital and 3-methylcholanthrene) when they were injected i.p. into rats once daily for 5 days [34]. Notably, when a higher dose of TCDD (100 µg/kg b.w.) was given to the animals for 8 weeks, it did not produce a greater induction of the hepatic E₂-metabolizing enzyme activity in these animals, but instead the levels of enzyme activity were significantly lower than what was seen with a lower dose of TCDD (10 µg/kg b.w.).

It is known that TCDD is a potent inducer of hepatic CYP1A1 and CYP1A2, and the induction is believed to be mediated by the hepatic AhR [4,20]. CYP1A2 is one of the major CYP isoforms present in liver, and it also has high catalytic activity for the 2- and 4-hydroxylation of E₂ [21,61]. Therefore, the nearly identical pattern of induction of the hepatic microsomal activity for E₂ 2- and 4-hydroxylation by TCDD appears to be in line with a predominant induction of the hepatic CYP1A2.

The animals receiving the HF diet for 8 or 16 weeks had a higher hepatic microsomal activity for the 2- and 4-hydroxylation of E₂ than animals fed the control diet. Interestingly, the inducing effect of TCDD on hepatic microsomal E₂-metabolizing activity was mostly abolished in animals fed a HF diet. This observation is rather unexpected, and the mechanism is not understood at present. It is speculated that the activation of certain PPAR signaling pathways by fatty acids may be involved in alterations of the AhR-mediated regulation of certain estrogen-metabolizing enzymes.

One of the marked changes in animals treated with TCDD is the increased hepatic activity for the *O*-methylation of 2-OH-E₂ and 4-OH-E₂ (up to 100% over the control values). Earlier studies have shown that COMT was not easily induced either *in vitro* or *in vivo* by various chemicals. Although the mechanism of COMT induction by TCDD treatment is not fully understood, it is possible that the AhR system may be involved, by transcriptionally activating the expression of the hepatic COMT gene. As suggested in a number of earlier studies [23], COMT is an important Phase II conjugation enzyme involved in catechol estrogen metabolism, and an increase in the hepatic enzyme activity for the biosynthesis of mono-methylated estrogens (in particular 2-methoxyestradiol) may be protective against estrogen-induced hormonal cancers [23]. In addition, higher levels of hepatic COMT activity would also contribute to enhanced detoxification of the endogenously-formed catechol estrogen metabolites (such as 4-hydroxy-E₂) *in vivo*, thereby jointly contributing to a reduced hormonal cancer risk.

Another major change in animals treated with TCDD is the decrease of the liver microsomal activity for the hydrolysis of E₁-3-sulfate. Changes of a similar magnitude were observed in animals fed either a control diet or a HF diet. It is known that E₁-3-sulfate is an important estrogen conjugate that has a relatively long half-life in circulation. This estrogen conjugate can be readily taken up by estrogen target cells via an active transport mechanism [62,63], and the subsequent hydrolysis mediated by sulfatases to release the hormonally-active parent hormone E₁ represents an important pathway for the biosynthesis of active estrogens in the body. Therefore, it is suggested that a marked decrease of hepatic sulfatase activity may subsequently reduce the body's overall estrogenic exposure and stimulation.

Conclusions

Administration of TCDD at a higher dose (100 µg/kg b.w.) once every 2 weeks for 8 weeks increased the body weight by 46% in the HF diet-fed animals, but this effect was not observed at two lower doses of TCDD (1 and 10 µg/kg b.w.) or animals fed a control diet. This is the first observation suggesting that TCDD, a potent AhR agonist, may have an obesity-inducing

effect in animals fed a HF diet, but not in all animals fed a normal diet. While TCDD alone increased liver weight and decreased thymus weight in animals, these effects were enhanced by feeding animals a HF diet. The change in the uterine wet weight in animals treated with TCDD with or without a HF diet appeared to be less uniform.

Metabolism studies showed that TCDD administration at 1 or 10 µg/kg b.w. once every 2 weeks for 8 or 16 weeks increased the liver microsomal activity for the 2- and 4-hydroxylation of E₂ in animals fed a control diet, but surprisingly not in animals fed a HF diet (which is different from TCDD's effect on liver weight). A higher dose of TCDD (at 100 µg/kg b.w.) showed a markedly weaker effect than the 10 µg/kg b.w. dose. Treatment with TCDD at 1–100 µg/kg b.w. for 8 or 16 weeks caused a dose-dependent increase in the hepatic activity for the *O*-methylation of catechol estrogens in both control and HF diet-fed animals, and it also decreased the levels of liver microsomal activity for the hydrolysis of E₁-3-sulfate. TCDD did not significantly affect the hepatic enzyme activity for the glucuronidation or esterification of endogenous E₂ and E₁. It is suggested that changes in hepatic estrogen-metabolizing enzyme activity in TCDD-treated, control diet-fed animals will beneficially alter the profiles of endogenous estrogen metabolites by increasing the formation of 2-methoxyestradiol (resulting from increased estrogen 2-hydroxylation plus increased *O*-methylation), along with a decreased estrogen biosynthesis from the sulfatase-mediated desulfation of endogenous estrogen-3-sulfates. It is believed that these changes in endogenous estrogen metabolism may jointly contribute to a reduced incidence of estrogen-associated tumorigenesis in various target organs of animals chronically treated with TCDD.

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Figure 1.

Effect of TCDD administration for 8 or 16 weeks on body weight (panels A–D), liver weight (panels E–H), thymus weight (panels I–L), and uterus weight (panels M–P) of female C3H/HeN mice fed a control diet or a HF diet. The animals (5–6 animals per group) were injected i.p. with TCDD at 1, 10, or 100 $\mu\text{g/kg b.w.}$ (dissolved in 100 μL corn oil) once every two weeks for 8 weeks or at 1 or 10 $\mu\text{g/kg b.w.}$ for 16 weeks; the control animals were injected the same volume of corn oil alone. Each value is the mean $\pm$ S.D. ($N = 5-6$). An asterisk placed on top of a bar denotes that the difference is statistically significant ($P < 0.05$, Tukey's test) when it was compared to the control (without TCDD treatment).

Figure 2.

Effect of TCDD administration for 8 or 16 weeks on the NADPH-dependent 2- and 4-hydroxylation of E₂ by liver microsomes of female C3H/HeN mice. The animals (5 – 6 animals per group) were injected i.p. with TCDD at 1, 10, or 100 µg/kg b.w. (dissolved in 100 µL corn oil) once every two weeks for 8 weeks or at 1 or 10 µg/kg b.w. for 16 weeks; the control animals were injected the same volume of corn oil alone. The collected livers from 5 – 6 animals in each treatment group were randomly pooled into two batches (with 2 – 3 livers/batch), and thus two separate batches of liver microsomes were prepared for each treatment group. The assay conditions and the measurement of the catechol estrogens by HPLC were described in the *Materials and Methods* section. Each bar represents the mean ± S.D. of triplicate measurements of each pooled microsomal preparation. An asterisk placed on top of a bar denotes that the difference is statistically significant ($P < 0.05$, Student's *t*-test) when it was compared to any of the two control values (without TCDD treatment).

Figure 3.

Effect of TCDD administration for 8 or 16 weeks on the *O*-methylation of 2-OHE₂ and 4-OH-E₂ by liver cytosol of female C3H/HeN mice. The TCDD treatment was the same as described in the legend to Figure 2. The collected livers from 5 – 6 animals in each treatment group were randomly pooled into two batches (with 2 – 3 livers/batch), and thus two separate batches of liver cytosols were prepared for each treatment group. The assay conditions and the measurement of methylated catechol estrogen metabolites were described in the *Materials and Methods* section. Each bar represents the mean $\pm$ S.D. of triplicate measurements of each pooled cytosol preparation. An asterisk placed on top of a bar denotes that the difference is statistically significant ($P < 0.05$, Student's *t*-test) when it was compared to any of the two control values (without TCDD treatment).

Figure 4.

Effect of TCDD administration for 8 or 16 weeks on the formation of E₂-17β-oleate from E₂ by liver microsomes of female C3H/HeN mice. The TCDD treatment and preparation of liver microsomes were the same as described in the legend to Figure 2. The assay conditions and the HPLC measurement of the E₂-17β-oleate were described in the *Materials and Methods* section. Each bar represents the mean ± S.D. of triplicate measurements of the microsomes prepared from 2 to 3 pooled liver samples. An asterisk placed on top of a bar denotes that the difference is statistically significant ($P < 0.05$, Student's *t*-test) when it was compared to any of the two control values (without TCDD treatment).

Figure 5.

Effect of TCDD administration for 8 or 16 weeks on the UDPGA-dependent glucuronidation of E₂ by liver microsomes of female C3H/HeN mice. The TCDD treatment and preparation of liver microsomes were the same as described in the legend to Figure 2. The assay conditions and the measurement of conjugated E₂ were described in the *Materials and Methods* section. Each bar represents the mean $\pm$ S.D. of triplicate measurements of the microsomes prepared from 2 to 3 pooled liver samples. An asterisk placed on top of a bar denotes that the difference is statistically significant ($P < 0.05$, Student's *t*-test) when it was compared to any of the two control values (without TCDD treatment).

Figure 6.

Effect of TCDD administration for 8 or 16 weeks on the formation of E₁ from E₁-3-sulfate by liver microsomes of female C3H/HeN mice. The TCDD treatment and preparation of liver microsomes were the same as described in the legend to Figure 2. The assay conditions and the measurement of the released E₁ were described in the *Materials and Methods* section. Each bar represents the mean $\pm$ S.D. of triplicate measurements of the microsomes prepared from 2 to 3 pooled liver samples. An asterisk placed on top of a bar denotes that the difference is statistically significant ($P < 0.05$, Student's *t*-test) when it was compared to any of the two control values (without TCDD treatment).

Figure 7.

Effect of a HF diet on animal body and organ weight and also on the activity of various hepatic estrogen-metabolizing enzymes. The female C3H/HeN mice were fed a HF diet for 8 or 16 weeks and injected with a vehicle (corn oil, 100 μ L) once every two weeks. For the body and organ weight, each bar represents the mean $\pm$ S.D. ($N = 5 - 6$ animals/group). For the estrogen-metabolizing enzyme activities, each bar represents the mean $\pm$ S.D. of triplicate measurements of the subcellular fractions prepared from pooled liver samples. An asterisk placed on top of a bar denotes that the difference is statistically significant ($P < 0.05$, Student's test) when it was compared to any of the two control values.

Table 1

The composition of the semi-synthetic AIN-76A control diet and the HF diet.

Ingredient	AIN-76A diet (D10011) (Control diet)		High-fat diet (D12370) (Newmark stress diet B)	
	grams	kcal	grams	kcal
Casein, 30 mesh	200	800	240	960
DL-Methionine	3	12	3.6	14
Corn starch	150	600	150	600
Sucrose	500	2000	294.2	1177
Cellulose, BW200	50	0	60	0
Corn oil	50	450	200	1800
Ethoxyquin	0.01	0	0.01	0
Salt mix S10001	35	0		
Salt mix S10001A			21	0
Mono-sodium phosphate			7.98	0
Mono-potassium phosphate			7.91	0
Calcium carbonate			0.88	0
Vitamin mix V10001	10	40		
Vitamin mix V13201			12	48
Vitamin D ₃ , 100,000 IU/gram			0.002	0
Choline bitartrate	2	0	2.4	0
Total	1000.01	3902	1000	4599